University of Aveiro Department of Chemistry
2015

RAQUEL SOFIA RIBEIRO LOPES

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química – Ramo Química Analítica e Qualidade, realizada sob a orientação científica do Doutor Armando da Costa Duarte, Professor Catedrático do Departamento de Química da Universidade de Aveiro e da Doutora Regina Maria Brandão de Oliveira Duarte, Investigadora em Pós-Doutoramento do CICECO – Instituto de Materiais de Aveiro e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

Associação Nacional de Investigadores em Ciência e Tecnologia

A Raquel Sofia Ribeiro Lopes usufruiu de uma **bolsa ANICT** para o desenvolvimento da Dissertação "Characterization of water-soluble organic compounds in bioaerosols by liquid chromatography and fluorescence spectroscopy".

Aos meus pais e irmã, pelo incansável apoio.

o júri

presidente

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palavras-chave

Bioaerossóis, compostos orgânicos solúveis em água, amostragem passiva, cromatografia líquida, separação cromatográfica de aminoácidos.

resumo

Nas últimas décadas, os efeitos da poluição atmosférica têm aumentado, essencialmente no caso das doenças nos seres humanos. De modo a ultrapassar este problema, a comunidade científica tem-se dedicado ao estudo dos componentes atmosféricos. Enquanto parte dos compostos orgânicos solúveis em água, os aminoácidos estão presentes na atmosfera como componentes dos organismos vivos, seres responsáveis por dispersarem doenças através do ar. A cromatografia líquida consiste numa das técnicas capazes de separar os diferentes aminoácidos entre si. Neste trabalho, com o intuito de separar os aminoácidos presentes em amostras de aerossóis recolhidas em Aveiro, foi avaliada a capacidade de separação de quatro colunas cromatográficas (Mixed-Mode WAX-1, Mixed-Mode HILIC-1, Luna HILIC e Luna C18) para quatro aminoácidos diferentes (ácido aspártico, lisina, glicina e triptofano) e a forma como a interação da fase estacionária destas colunas com os analitos é influenciada pela percentagem de solvente orgânico e pela presença/concentração do tampão.

Na coluna Mixed-Mode WAX-1, os cromatogramas dos diferentes aminoácidos revelaram que a separação não era eficiente, sendo os tempos de retenção bastante similares. No caso da lisina, na eluição a 80% (V/V) MeOH, os picos apareceram durante o volume morto. No caso da coluna Mixed-Mode HILIC-1, a variação da concentração do solvente orgânico não influenciou a eluição dos quatro aminoácidos em estudo. Considerando a coluna Luna HILIC, os tempos de retenção dos vários aminoácidos eram demasiados próximos para garantir uma separação entre os mesmos. Por fim, a coluna Luna C18 mostrou-se útil na separação de aminoácidos no modo gradiente, no qual a variação da constituição da fase móvel ocorreu em termos de volume do solvente orgânico (ACN), tendo sido esta a coluna utilizada para a separação das amostras reais. A fase móvel era constituída por água acidificada e ACN, e o gradiente consistia no seguinte programa: 0 – 2 min: 5% (V/V) ACN, 2 – 8 min: 5 – 2 % (V/V) ACN, 8 – 16 min: 2% (V/V) ACN, 16 – 20 min: 2 – 20 % (V/V) ACN, 20 – 35 min: 20 – 35 % (V/V) ACN.

As amostras de aerossóis foram obtidas com recurso a três amostradores passivos colocados em dois locais distintos de Aveiro, cada amostrador contendo dois filtros - um virado para cima e outro para baixo. Após a amostragem, a matéria orgânica solúvel em água foi extraída com recurso a dissolução em água ultra-pura, banho de ultrassons e filtração. Os filtrados recolhidos foram diluídos em água acidificada para a separação cromatográfica. Os resultados da cromatografia líquida evidenciaram a presença de aminoácidos, não tendo sido possível efetuar a sua identificação individual. Os cromatogramas e os espetros de fluorescência indicaram a existência de padrões: as amostras correspondentes aos filtros de cima possuíam sinais mais intensos, indicando que os filtros de cima recolheram maior quantidade de matéria orgânica.

keywords

Bioaerosols, water-soluble organic carbon, passive sampling, liquid chromatography, amino acids chromatographic separation.

abstract

In the last decades, the effects of the air pollution have been increasing, especially in the case of the human health diseases. In order to overcome this problem, scientists have been studying the components of the air. As a part of water-soluble organic compounds, amino acids are present in the atmospheric environment as components of diverse living organisms which can be responsible for spreading diseases through the air. Liquid chromatography is one technique capable of distinguish the different amino acids from each other. In this work, aiming at separating the amino acids found in the aerosols samples collected in Aveiro, the ability of four columns (Mixed-Mode WAX-1, Mixed-Mode HILIC-1, Luna HILIC and Luna C18) to separate four amino acids (aspartic acid, lysine, glycine and tryptophan) and the way the interaction of the stationary phases of the columns with the analytes is influenced by organic solvent concentration and presence/concentration of the buffer, are being assessed.

In the Mixed-Mode WAX-1 column, the chromatograms of the distinct amino acids revealed the separation was not efficient, since the retention times were very similar. In the case of lysine, in the elution with 80% (V/V) MeOH, the peaks appeared during the volume void. In the Mixed-Mode HILIC-1 column, the variation of the organic solvent concentration did not affect the elution of the four studied amino acids. Considering the Luna HILIC column, the retention times of the amino acids were too close to each other to ensure a separation among each other. Lastly, the Luna C18 column revealed to be useful to separate amino acids in a gradient mode, being the variation of the mobile phase composition in the organic solvent concentration (ACN). Luna C18 was the column used to separate the amino acids in the real samples and the mobile phase had acidified water and ACN. The gradient consisted in the following program: 0 – 2 min: 5% (V/V) ACN, 2 – 8 min: 5 – 2 % (V/V) ACN, 8 – 16 min: 2% (V/V) ACN, 16 – 20 min: 2 – 20 % (V/V) ACN, 20 – 35 min: 20 – 35 % (V/V) ACN.

The aerosols samples were collected by using three passive samplers placed in two different locations in Aveiro and each sampler had two filters - one faced up and the other faced down. After the sampling, the water-soluble organic compounds was extracted by dissolution in ultra-pure water, sonication bath and filtration. The resulting filtered solutions were diluted in acidified water for the chromatographic separation. The results from liquid chromatography revealed the presence of the amino acids, although it was not possible to identify each one of them individually. The chromatograms and the fluorescence spectra showed the existence of some patterns: the samples that correspond to the up filters had more intense peaks and signals, revealing that the up filters collected more organic matter.

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ACN – acetonitrile

CE – capillary electrophoresis

CSPs – chiral stationary phases

EEM – excitation-emission matrix

ELSD – evaporative light-scattering detector

FID – flame ionization detector

GABA – gamma-aminobutyric acid

GC – gas chromatography

HILIC – hydrophilic interaction liquid chromatography

LIF – laser-induced fluorescence

MeOH – methanol

MS – mass spectrometry

MSD – mass selective detector

PDFOA – pentadecafluorooctanoic acid

RP – reversed-phase

SRM – standard reference material

TDFHA – tridecafluoroheptanoic acid

WAX– weak anionic exchange

WSOC – water-soluble organic carbon

I

Introduction: basic concepts of aerosols and liquid chromatography

1. Aerosols: general considerations

Through the years, the interest in developing precise technologies and methods able to detect and identify atmospheric particles has been growing (Després *et al.*, 2012). Atmospheric particles can be emitted into the atmosphere from natural or anthropogenic sources and they can be formed *in-situ* in the atmosphere through secondary formation processes - aerosols can be primary or secondary, regarding their formation processes. According to Hinds (1999), aerosols consist in colloidal systems of liquid or solid particles suspended in a gas. Primary particles are directly released into the atmosphere. The products of biomass burning, volcanic eruptions, combustion of fossil fuels and wind-driven particles of soil dust, sea salt and biological materials are some examples of primary aerosols (Hallquist *et al.*, 2009). On the other hand, secondary particles are the result of volatile and semi-volatile organic species transformations occurring in the atmosphere and involving gas-to-particle conversion processes, including condensation, nucleation and heterogeneous and multiphase chemical reactions. During these processes, bioaerosols suffer degradation and lose their biologic functions, producing inorganic secondary aerosols particles, such as sulfate (SO_4^{2-}), nitrate (NO_3^-) and ammonium (NH_4^+), which are products of the conversion of the sulfur dioxide (SO_2), nitrogen dioxide (NO_2) and ammonia (NH_3) gases into the particulate phase, respectively (Goldstein & Galbally, 2007; Kroll & Seinfeld, 2008).

When atmospheric particles have a biological origin, such as bacteria, fungi, viruses, debris, dust mites, toxins, spores, cells, pollen, animal or plant organic matter and sub products of biological activities, they are called **bioaerosols**, and their size can vary from 0.5 to 100 μm (Després *et al.*, 2012; Wathes & Cox, 1995).

1.1 - Bioaerosols: a particular case of aerosols

Bioaerosols are present both in outdoor and indoor environments, in a wide range of concentrations, and they may be aerosolized from diverse sources: soil, vegetation, water and living organisms. Commonly, bioaerosols show a considerable diversity in dimension, shape and electric charge. Furthermore, as reviewed by Agranovski (2010), it is often significant to make the distinction between viable and non-viable bioaerosols particles: the first one is referred to microbial cells capable of reproducing or having a metabolic behavior; and the second one is related to organisms unable to reproduce. According to Gao *et al.* (2009), only less than 10% of the airborne bacteria particles are viable and the higher viabilities have been detected in fungal spores. Considering the importance of the biological activity of bioaerosols due to the possibility of new microorganisms formation, bioaerosols characterization is different from the one traditionally applied on inert aerosols (Agranovski, 2010).

As mentioned by Agranovski (2010), the most common and studied bioaerosols particles are bacteria, pollen, viruses and fungi. Viruses are unique since they have the ability to reproduce but only inside a host cell. However, there is still an absence of information about their shape, size and density. Recently, there has been some evolution in the knowledge regarding the way airborne viral particles travel in the air. It used to be believed these bioaerosols would only survive as airborne if they were attached to a larger particle, in a highly humid environment. Nowadays, due to studies such as the amount of time a laboratory-generated single viruses is able to survive in the air (Lighthart and Shaffer, 1997) and the transmission of the influenza virus (Elbert *et al.*, 2007), it is known that viruses can be diffused through the air without extreme conditions, which can be harmful if the airborne transmission of viral infections increases.

Bacteria are organisms with only one cell which can have various shapes, including spiral, spherical and rod-shaped, and they can be carried through the air by other aerosols like water droplets, fragments of animal skin and plant material (Putaud *et al.*, 2004; Lee *et al.*, 2008). These prokaryote organisms tend to grow in colonies and when aerosolized they frequently aggregate to other materials or to each other in clusters or chains (Conte and Weber, 2002). Both animals and plants can be seriously ill due to airborne bacteria particles. Some bacteria can generate spores related to specific human

health diseases such as respiratory allergy and asthma resulting from occupational exposures (Kourtchev *et al.*, 2008).

Fungi are spread in the atmosphere due to the release of spores and they are adapted to several atmospheric environments. According to Lee *et al.* (2007, 2008), fungi are extremely resistant to high and low temperatures, low humidity and ultraviolet radiation and they can become aerosolized as either individual or agglomerated spores. Fungi airborne particles are associated with allergic reactions, allergic rhinitis and asthma.

Plants produce pollen grains in a significant quantity wherein the characteristics of the airborne pollen are related to the plant that create it. So, particles from different plants diverge in size, shape and surface structure. There is a lack of information related to the aerodynamic diameters of pollen particles, although it is known that the physical size is in the range of 10 to 100 µm. However, numerous types of pollen grains have 25 – 50 µm, values larger than the breathable size fraction (Reponen *et al.*, 2009). Thus, human health effects are associated with smaller and not so usual pollen fragments that may contain allergens (Wathes and Cox, 1995; Miguel *et al.*, 2006).

1.2 - Bulk deposition of aerosols

Every atmospheric particle participates in exchanges with outdoor surfaces, in processes including deposition or removal from the atmosphere and re-suspension or re--entrainment from underlying surfaces (Wathes and Cox, 1995). Removal methods may occur via either wet or dry deposition or both ways combined, as depicted in **Figure 1**.

Dry deposition consists in the direct interaction of a particle with a surface, in a continuous process, through diffusion and/or sedimentation. Wet deposition embraces the removal of material from the atmosphere in any falling particle, such as raindrops, snowflakes or hailstones. After deposition, aerosols may experience the process of resuspension or reemission, which can result in the previously deposited material being reemitted into the atmosphere (Després *et al.*, 2012).

Dry deposition is the main removal mechanism for particles with a diameter equal or higher than 10 μm. The atmospheric sedimenting particles are the ones that hit a barrier, usually the ground, and stop their movement. They can be deposited via a dry or wet deposition method. On the other hand, there are other atmospheric constituents that do not stop their movement in the atmosphere, not hitting any barrier: the non-sedimenting constituents (Després *et al.*, 2012).

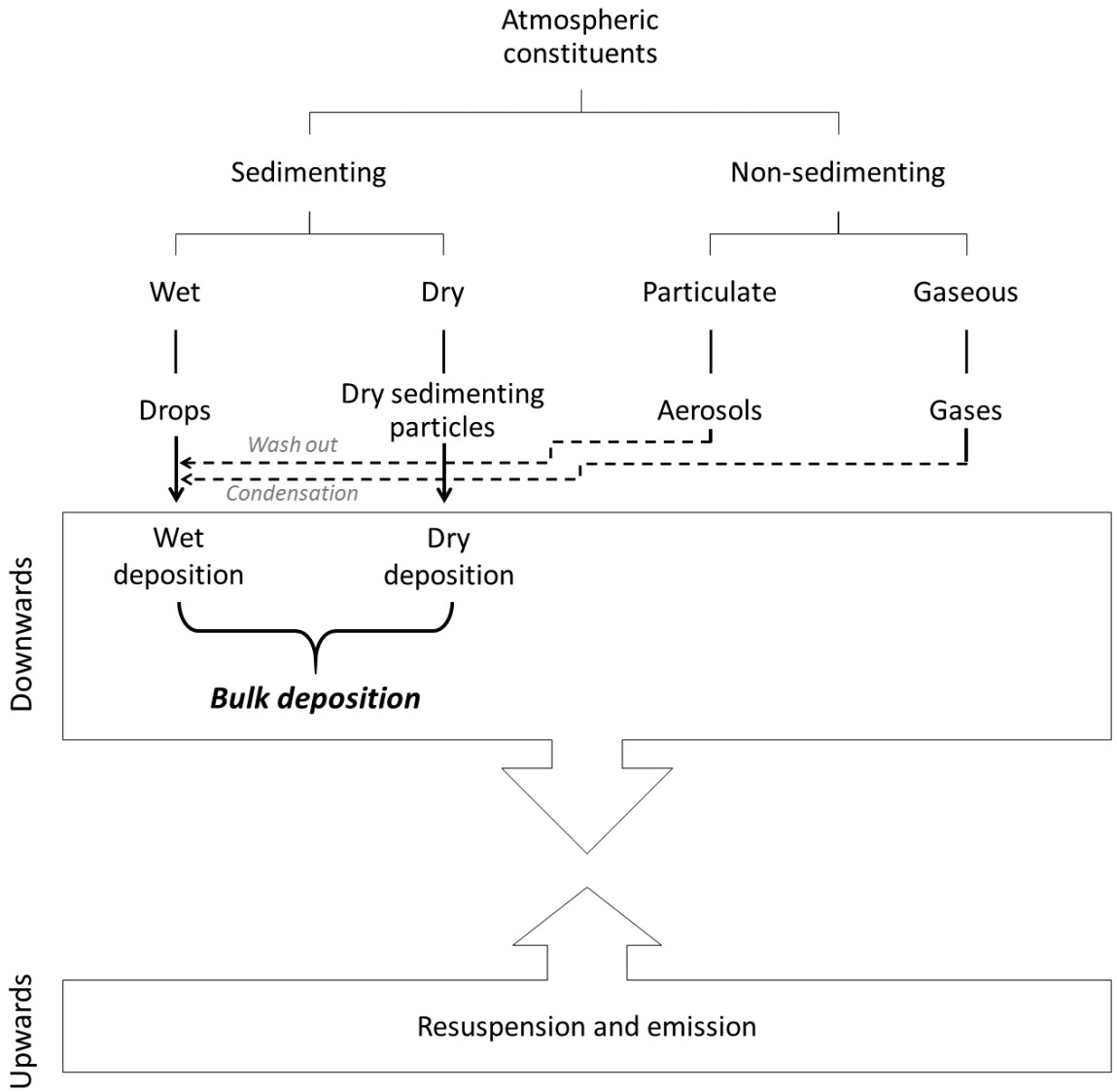


Figure 1 Processes of atmosphere-surface exchanges related to aerosols (adapted from Dämmgen *et al.*, 2005).

In the non-sedimenting situation, particles can only be removed via wet deposition. When these particles are in the gaseous state, they can be removed by wet deposition after a condensation process, turning in to drops. In the case where the non-

-sedimenting particles include components in the solid state, they are removed from the atmosphere also via wet deposition, though a wash out process, as shown in **Figure 1**.

When dry deposition procedures are adopted, particles are studied by their aerodynamic diameter – which is defined as the diameter of a spherical particle with a unit density and with the same terminal velocity in the air as the aerosols' particle in question (Hinds, 1999). Since biological particles frequently have a complex structure with irregular surfaces, internal pores and asymmetric shape, their aerodynamic and physical sizes may not match perfectly. Therefore, aerodynamic diameter is widely used in bioaerosols analysis (Tong and Lighthart, 2000).

In the case of particles with an aerodynamic diameter within 0.1 – 10 μm, deposition by precipitation is the most efficient removal method, removing a large percentage of particles from the air in many situations, even if it is a single rain event (McDonald, 1962). Dry deposition, on the other hand, is not well understood due to complexities regarding measuring gaseous concentrations and calculating deposition velocities necessary for quantifying dry deposition rates (Elliott *et al.*, 2009).

As shown in **Figure 1**, bulk deposition is considered as the whole amount of wet and dry deposition collected during sampling. In practical terms, this amount can have some bias due to continuously deposition of gases and fine aerosols to the sampler. Therefore, bulk deposition is frequently known as the total deposition of particles to a constantly opened sampler. During sampling preparation, it is crucial to be aware of the particles that accidentally can enter the sampler. For example, only the material from the surrounding ecosystem must be a part of the sample, which may not always happen due to turbulent deposition. Besides, the sample may also include the contribution from sedimentation particles that have been resuspended by the wind from the adjacent environment. Since these sampling artifacts can easily occur during aerosol collection, it is extremely important to choose well the sampler location and height above ground (Dämmgen *et al.*, 2005).

1.3 - Strategies for sampling (bio)aerosols

Relatively to air microorganisms monitoring, there are two main sampling methods: the active and the passive sampling. The theory for passive sampling techniques was introduced by Palmes and Gunnison (1973), who developed a diffusive sampler to determinate the presence of SO_2 in the atmosphere. Passive samplers are devices capable of collecting atmospheric gases or vapors, via a controlled rate by physical processes, such as diffusion or permeation, not involving active movement of the air through the sampler (Brown *et al.*, 1984). Firstly, these samplers were invented in order to be used in indoor studies due to humans' exposure to high levels of pollutants in working areas. Meanwhile, since the 80's, the samplers started to be applied to outdoor environmental research, where the pollutants concentrations are extremely lower. Nevertheless, these investigations are challenging, since there is the influence of the meteorological conditions (Palmes *et al.*, 1976; Brown *et al.*, 1984).

Comparatively to active samplers, the passive samplers have been gaining more supporters for gases and vapors sampling since they are simpler, cheaper, smaller, lighter, easier to carry to the sampling site, and do not need electric power or periodic calibrations. Besides, it is not necessary to measure the volume of the sampled air or the every-day presence of a technician in the sampling location, and the sample is more representative of the natural process of deposition (Rose and Perkins, 1982). These samplers are particularly useful in determining the spatial distribution of the atmospheric gases concentrations in large areas, as well as monitoring studies in remote zones and providing data for modeling and comparisons with air quality long term standards. Despite of the aforementioned advantages of these devices, they are not always completely reliable: the passive samplers do not provide instantaneous concentrations but mean values every week or month, thus not allowing obtaining the sampling rate and, consequently, it is not possible to distinguish the transitional moments between highest and lowest concentrations in a certain period (Shooter *et al.*, 1995). Furthermore, they are not as sophisticated as the active samplers (Cruz and Campos, 2002).

In these passive samplers, inertial forces along with the external wind and gravity, provide the transportation of aerosol particles to collectors' surfaces. Although there was no concept of "passive sampler" at the time, Oren Durham (1946) planned a very simple passive sampling apparatus for atmospheric particles involving essentially the

gravitational deposition of those aerosols onto an oil-coated flat slide. However, there was some interference related to the falling of debris and rain. Therefore, the author set a cover in order to direct the collected aerosol into the region of the sampling surface so the sample would experiment the influence of the horizontal wind. Some decades later, in 1974, as mentioned by Vicent (2007), Tauber improved the original idea from Durham and designed a more sophisticated passive sampler, specifically to collect large pollen grains. As shown in **Figure 2**, aerosols enter the sampler through the circular opening at the top of the cylindrical container by a combination of inertial and gravitational forces. The sample is retained on the glycerol-coated surface on the bottom of the sampler (Vincent, 2007).

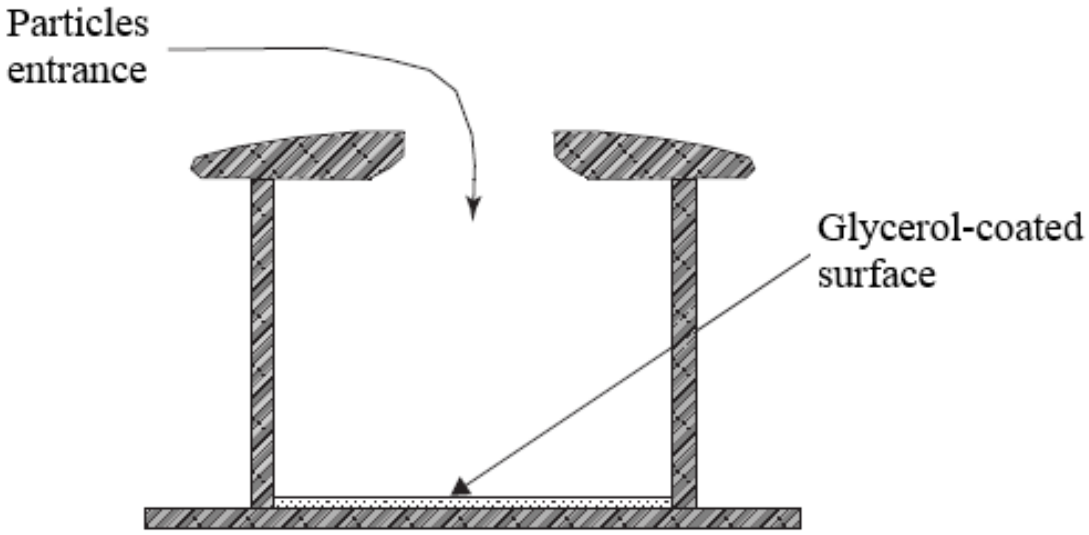


Figure 2 Illustration of the principle of operation of the Tauber pollen sampler (adapted from Vincent, 2007).

1.4 - Amino acids as proxies of bioaerosols

According to some authors, there are several methods with the ability to detect the presence of bacteria, pollen and fungal spores in atmospheric particles, such as the use of chemical biomarkers (Di Filippo *et al.*, 2013), traps (Ong *et al.*, 1995; Ho *et al.*, 2005), molecular biology techniques (Womiloju *et al.*, 2003; Lee *et al.*, 2006) or microorganism sampling, growing and counting (Bauer *et al.*, 2002). The aforementioned biological

methods are difficult to apply and only give partial information since they are limited to the biological component under study. These techniques are applied on amino acids and proteins, important constituents of bioaerosols, allowing the use of these biomolecules as an index of biological occurrence in the atmosphere. Plants, animals, biomass burning and degradation products of peptides and proteins through enzymatic and photo-catalytic reactions in the atmosphere release amino acids directly to the air, which make them responsible for free amino acids contribution to airborne particles (Di Filippo *et al.*, 2014).

Amino acids are composed by a carboxyl and an amino functional groups, an R group and a hydrogen atom, bonded to the same carbon atom (α carbon). All of the 20 common amino acids are α-amino acids and they differ from each other in their **R group**, also known as side chain. The fact that the α carbon is bonded to four distinct groups makes it a chiral atom, except in the case of glycine since the R group is an hydrogen atom. Having only one chiral center, 19 of the 20 common amino acids are chiral compounds (Nelson and Cox, 2004).

Although amino acids have a common structure, as shown in **Figure 3**, except in the case of proline, the side chains vary in size, polarity, structure and electric charge, some of the properties capable of influence the molecules solubility in water (Nelson and Cox, 2004).

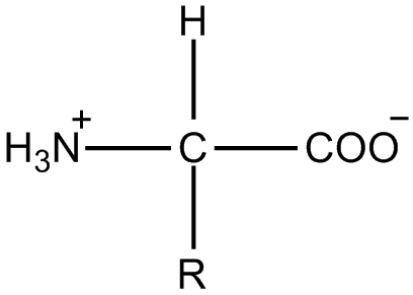


Figure 3 General structure of an amino acid; the R group designates the side chain.

As a consequence of the tetrahedral arrangement of the bonding orbitals around the carbon atom, the four diverse groups may be organized in two unique spatial adjustments and, thus, amino acids are stereoisomers: the same compound may have divergent spatial arrangements of its' atoms, although the molecules possess the same atoms and sequence of bonds. It is not possible to convert one molecule's stereoisomer into the other without breaking the inherent bonds. Since the stereoisomers of amino acids are non-superimposable mirror images of each other, they are called **enantiomers**. Thus,

a special nomenclature has been implemented to describe the absolute configuration of the four substituents of an optical isomer (enantiomer): the **D/L system**, a convention proposed by Emil Fischer in 1891. According to this system, the enantiomer is named by analogy to glyceraldehyde, shown in **Figure 4 – a)**, consistent with which glyceraldehyde isomer it comes from. Therefore, if an enantiomer comes from D-glyceraldehyde that isomer it also labeled as the *D*-form (Nelson and Cox, 2004).

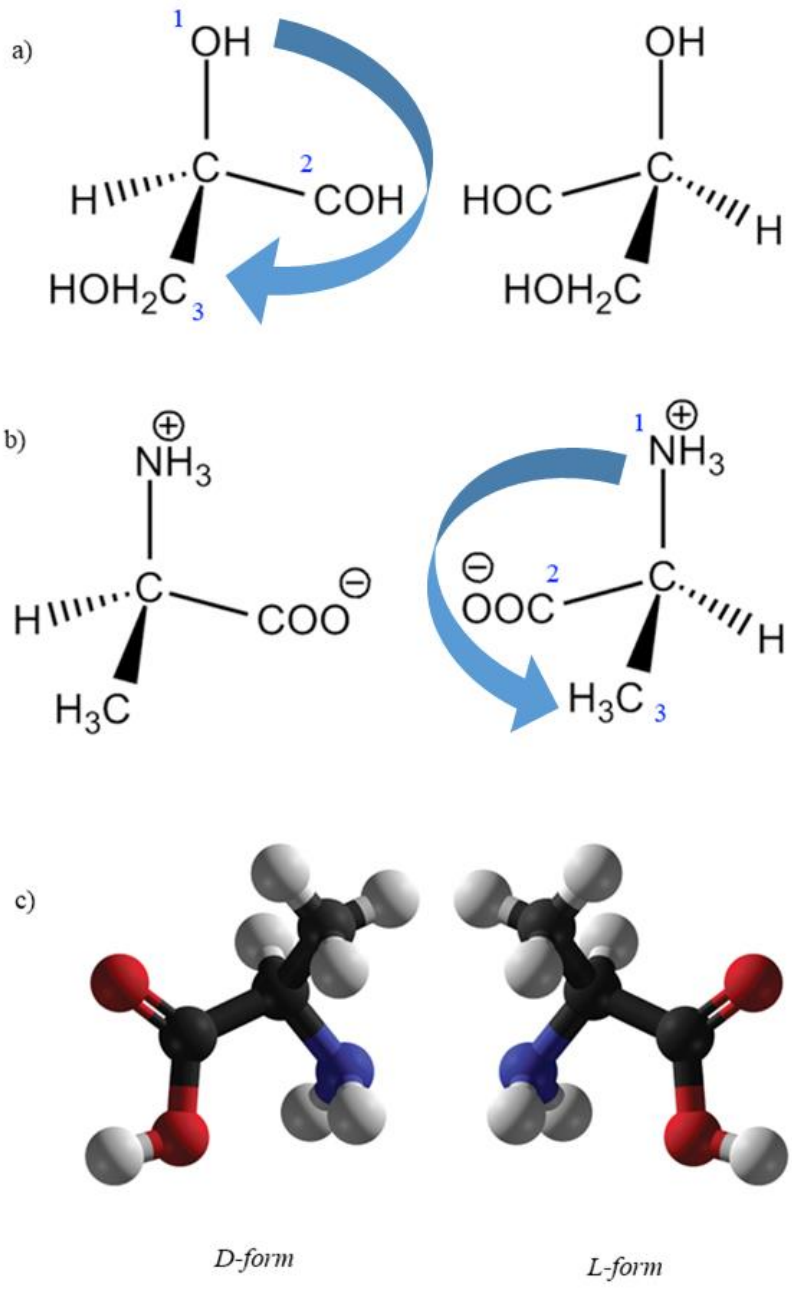


Figure 4 Enantiomers of **a)** glyceraldehyde and **b)** alanine. In both cases, the chiral atom is in the centre of the projection, the *D*- enantiomer is on the left and the *L*- on the right side. **c)** Three-dimensional representation of D-alanine and L-alanine, showing the mirror effect between the enantiomers.

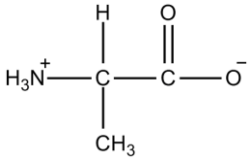
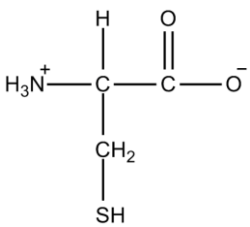
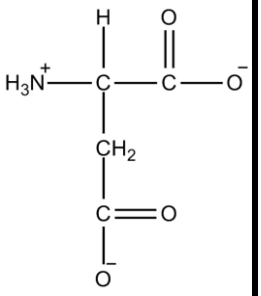
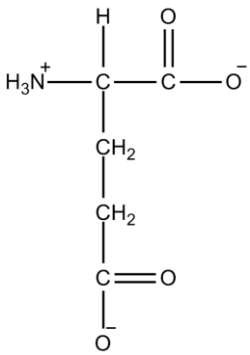
Besides the relation to the enantiomers of glyceraldehyde, it is possible to name the amino acids according to the atomic number of the atom bonded to the chiral center. The atom with the highest atomic number is the number 1 atom; the atom with the second highest atomic number corresponds to the atom number 2; and so on. If the chiral center is bonded to more than one unit of the same atom, the rule above is applied to the atoms of that group. For example, in the case of alanine (**Figure 4 – b) and c)**), there are two carbons bonded to the chiral center: a COO^- and a CH_3 group. Therefore, the atoms that will be compared are oxygen and hydrogen. The atom that is used for the comparison is always the one with the highest atomic number, as for the atoms S and O in the groups C-HHSH and C-OOH, the atoms S and O are the ones being compared. If the atoms are the same but with different bonds then the type of bond influences the result: a double bond (C=O) has priority over a single bond (C-O). After matching the groups with their priority number, the direction is obtained according to the increasing numerical order: 1 → 2 → 3. If the groups are arranged in the clockwise way, the amino acid is in its *D*-form (dextrorotary). On the other hand, if the spatial distribution is counterclockwise, the enantiomer is labeled as *L*- (levorotary). Although this is a simple procedure, it is crucial the hydrogen atom, component of all the amino acids, is away from the viewer in the tri-dimensional conformation, in order to name correctly the enantiomers. If the hydrogen atom is close to the viewer, the result of the nomenclature process is inversed: the *D*- is related to the counterclockwise direction and the *L*- becomes the arrangement of the clockwise way (Nelson and Cox, 2004).

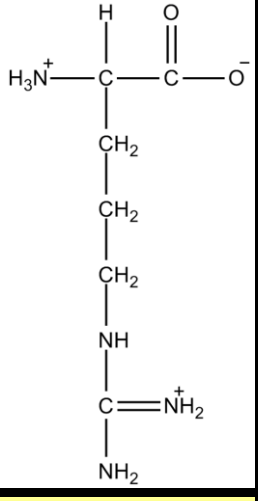
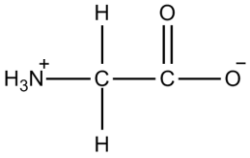
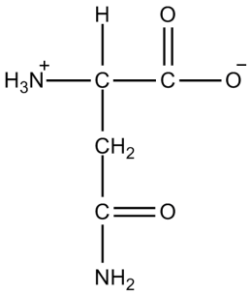
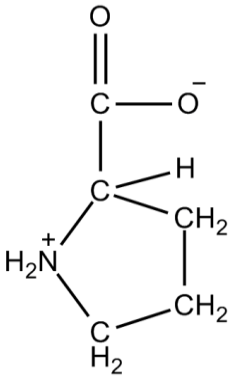
In Nature, the fact that amino acids are enantiomers has a significant role in biosynthesis: the amino acids residues present in proteins are exclusively *L*-enantiomers. When chiral compounds are produced by a conventional chemical reaction, the obtained result is a racemic mixture of both *D*- and *L*- isomers. Distinguish and/or separate these two different molecules is a difficult task to achieve. However, if the product is formed by a living organism, *D*- and *L*- enantiomers are totally dissimilar. Usually, the formation of stable and consistent structures in proteins requires that only one of the enantiomers is a constituent. In order to keep the organism alive and functional, cells specifically synthetize the *L*- isomers, since the active sites of enzymes are asymmetric, causing that the reactions they catalyze to be stereospecific. Therefore, and considering that only *L*-forms of amino acids are used to produce proteins during biosynthesis, it is possible to state that there is a homochirality regarding living organisms. On the other hand, *D*-amino acids exist in cell walls of bacteria (Barbaro *et al.*, 2014a).

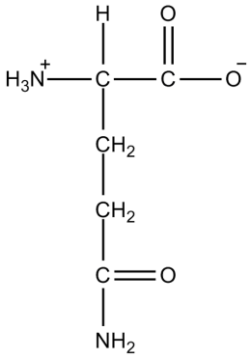
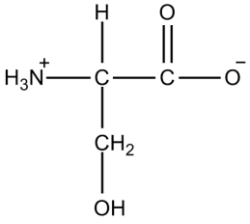
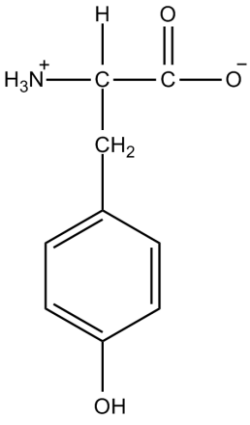
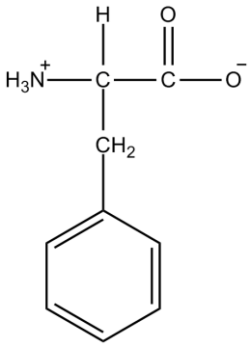
Furthermore, enantiomers have the same physical properties but they rotate polarized light in a divergent direction and they interact differently with distinct optical isomers of other compounds. All chiral compounds rotate the plane-polarized light, which means they are optically active (Nelson and Cox, 2004).

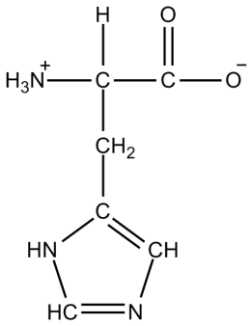
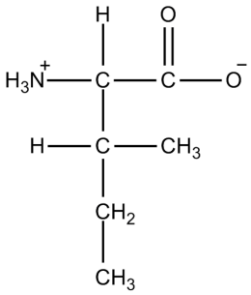
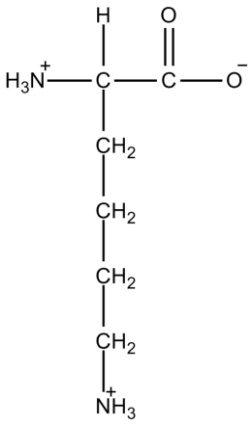
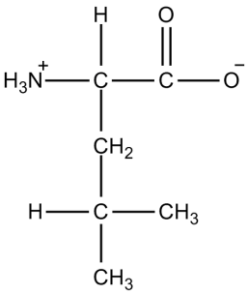
In order to indicate the sequence of the amino acids polymerized in proteins, the 20 common amino acids have been assigned three-letter abbreviations and one-letter symbol as shown in **Table 1**. Regarding human daily basics needs, there are three categories of amino acids: non-essential, conditionally non-essential and essential. In the table below, the non-essential amino acids are marked with green color (▬) and can be synthesized by the human organism. The conditionally non-essential amino acids, marked with yellow color (▬), are usually produced in our organism. However, some people are not capable of synthesize them in sufficient quantity, causing a necessity to include those amino acids in their diet. Finally, the essential amino acids, red color (▬), cannot be produced by the human organism, and consequently, they have to be included in our diet (Nelson and Cox, 2004).

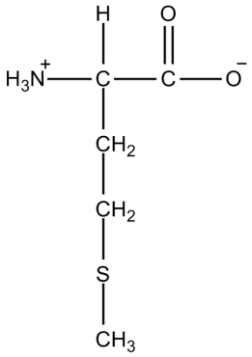
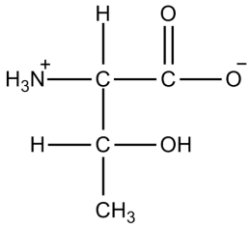
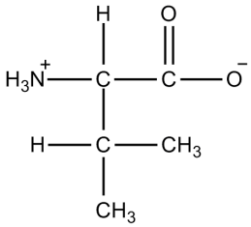
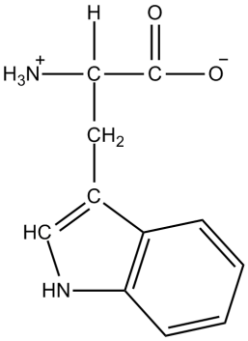
Table 1 Abbreviations and properties related to the twenty amino acids found in proteins (adapted from Nelson and Cox, 2004).

Amino acid	Alanine	Cysteine	Aspartic acid (aspartate)	Glutamic acid (glutamate)
Three-letter code	Ala	Cys	Asp	Glu
One-letter code	A	C	D	E
Molecular formula	$C_3H_7NO_2$	$C_3H_7NO_2S$	$C_4H_7NO_4$	$C_5H_9NO_4$
Molecular weight (g/mol)	89.0932	121.160	133.103	147.129
Molecular structure				

Amino acid	Arginine	Glycine	Asparagine	Proline
Three-letter code	Arg	Gly	Asn	Pro
One-letter code	R	G	N	P
Molecular formula	$C_6H_{14}N_4O_2$	$C_2H_5NO_2$	$C_4H_8N_2O_3$	$C_5H_9NO_2$
Molecular weight (g/mol)	174.201	75.0666	132.118	115.130
Molecular structure				

Amino acid	Glutamine	Serine	Tyrosine	Phenylalanine
Three-letter code	Gln	Ser	Tyr	Phe
One-letter code	Q	S	Y	F
Molecular formula	$C_5H_{10}N_2O_3$	$C_3H_7NO_3$	$C_9H_{11}NO_3$	$C_9H_{11}NO_2$
Molecular weight (g/mol)	146.144	105.093	181.189	165.189
Molecular structure				

Amino acid	Histidine	Isoleucine	Lysine	Leucine
Three-letter code	His	Ile	Lys	Leu
One-letter code	H	I	K	L
Molecular formula	$C_6H_9N_3O_2$	$C_6H_{13}NO_2$	$C_6H_{14}N_2O_2$	$C_6H_{13}NO_2$
Molecular weight (g/mol)	155.155	131.173	146.188	131.173
Molecular structure				

Amino acid	Methionine	Threonine	Valine	Tryptophan
Three-letter code	Met	Thr	Val	Trp
One-letter code	M	T	V	W
Molecular formula	$C_5H_{11}NO_2S$	$C_4H_9NO_3$	$C_5H_{11}NO_2$	$C_{11}H_{12}N_2O_2$
Molecular weight (g/mol)	149.211	119.119	117.146	204.225
Molecular structure				

The isomerism is not the only property that influences the analysis of the amino acids present in aerosols. Since the aim of this work is to separate amino acids enantiomers through liquid chromatography, there are other fundamental characteristics that play a role in the efficiency of this method, such as size, polarity, charge at pH 7.0 and pI (isoelectric point – pH value at which a certain molecule does not have electric charge), as shown in **Table 2**.

Although all of the 20 common amino acids are soluble in water, it is necessary to be aware of their affinity to this molecule. Therefore, it is also revealed in **Table 2** the hydropathy index - a scale combining hydrophobicity and hydrophilicity of R groups that can be used to measure the tendency of an amino acid to seek an aqueous environment (negative values) or a hydrophobic environment (positive values). These values were studied by Kyte and Doolittle (1982) and obtained by using a computer software that evaluates, progressively, the hydrophilicity and the hydrophobicity of a protein along its amino acids sequence. According to Kyte and Doolittle (1982) results, arginine is the amino acid with the higher tendency to interact with water while isoleucine is the most hydrophobic one. Considering the relation between hydropathy and the polarity of the molecule, it is possible to assert that arginine is the most polar amino acid while isoleucine is the most nonpolar one, in a group of 9 nonpolar and 11 polar amino acids. Although the software assigns numeric values to hydropathy, the results are not completely linear and need to be analyzed. In cases where amino acids are truly large or small, such as tryptophan and glycine, the software is not particularly accurate, and it assigns negative values (hydrophilic properties) to nonpolar molecules (Kyte and Doolittle, 1982).

Arginine is also the amino acid with the highest pH value at which it becomes electrically neutral (highest pI value). On the other hand, aspartic acid is the one with the lowest pI. Among the 20 amino acids, there are three with a positive electric charge at pH 7.0, the ones with a basic behavior: histidine, lysine and arginine. The molecules with a negative electric charge at pH 7.0 are the ones with an acidic behavior: aspartic acid and glutamate. The other 15 amino acids are neutral at pH 7.0. The largest molecule is tryptophan whereas the smallest is glycine (Nelson and Cox, 2004).

Table 2 Properties of the 20 common amino acids that influence the efficiency of the liquid chromatography separation process: size, polarity, electric charge at pH 7.0, behaviour at pH 7.0, pI and affinity to water (adapted from Nelson & Cox, 2004; Pommié *et al.*, 2004).

		Ala	**Cys**	**Asp**	**Glu**	**Gly**	**Asn**	**Pro**	**Gln**	**Arg**	**Ser**
Polarity	Polar			X	X		X		X	X	X
	Nonpolar	X	X			X		X			
Hydropathy index		1.8	2.5	-3.5	-3.5	-0.4	-3.5	1.6	-3.5	-4.5	-0.8
pI		6.01	5.07	2.77	3.22	5.97	5.41	6.48	5.65	10.76	5.68
Charge	Negative			X	X						
	Neutral	X	X			X	X	X	X		X
	Positive									X	
Volume	Large									X	
	Medium				X				X		
	Small	X	X	X		X	X	X			X
pH 7.0	Acidic			X	X						
	Neutral	X	X			X	X	X	X		X
	Basic									X	
		Tyr	**Phe**	**His**	**Ile**	**Lys**	**Leu**	**Met**	**Thr**	**Val**	**Trp**
Polarity	Polar	X		X		X			X		
	Nonpolar		X		X		X	X		X	X
Hydropathy index		-1.3	2.8	-3.2	4.5	-3.9	3.8	1.9	-0.7	4.2	-0.9
pI		5.66	5.48	7.59	6.02	9.74	5.98	5.74	5.87	5.97	5.89
Charge	Negative										
	Neutral	X	X		X		X	X	X	X	X
	Positive			X		X					
Volume	Large	X	X		X	X	X	X			X
	Medium			X						X	
	Small								X		
pH 7.0	Acidic										
	Neutral	X	X		X		X	X	X	X	X
	Basic			X		X					

1.5 - Methodologies for the analysis of amino acids in aerosols

Amino acids, free and combined, can be a component of **water-soluble organic carbon (WSOC)**, and their presence in the atmosphere has been studied for the last decades. This study is truly challenging due to their low ambient concentrations. Besides, proteins and peptides may undergo modifications through chemical and physical processes (Haan *et al*., 2009).

Although the quantification is a difficult task, amino acids extraction can be a simple practice according to the procedure followed by Matsumoto and Uematsu (2005). According to these authors, it is possible to separate 14 of the 20 common amino acids via an easy extraction method: sonication with pure water. Since this research work is focused on the extraction and separation of WSOC, and in amino acids in particular, Matsumoto and Uematsu's work appears to be a successful study using a simple extraction procedure.

Over the latest fifteen years, there have been studies in a wide range of procedures for extraction and analysis of amino acids in diverse matrices, from mural painting to standard reference material, using liquid chromatography. Examples of some of those studies are summarized in **Table 3,** considering the following characteristics: matrix, extraction, clean-up methods, separation and detection techniques, composition of the mobile and stationary phases, and the amino acids that were analyzed. As it is possible to observe in **Table 3**, the extraction method for amino acids can be complex and laborious such as in the works of Johnson and Pregitzer (2007), Zangrando *et al.* (2010) and Di Filippo *et al.* (2014), or simple such as in the works of Barbaro *et al.* (2011), Scalabrin *et al.* (2012) and Warren (2013). Concerning the clean-up process for the isolation of amino acids from other potential interfering analytes, Barbaro *et al.* (2011, 2014b), Warren (2008) and Zangrando *et al.* (2010) used a filtration step in their work. On the other hand, Di Filippo *et al.* (2014) and Buiarelli *et al.* (2013) prefered the solid phase extraction as a clean-up methodology to isolate the amino acids in their samples. Although the majority of the listed works (**Table 3**) has used a mass spectrometry as detector, some authors adopted a two-dimensional liquid chromatography method for the separation of amino acids, coupled to different detectors, namely UV-Vis (Mace *et al.*, 2003), flame ionization (Amelung & Zhang, 2001), fluorescence (Johnson & Pregitzer, 2007) and evaporative light scattering (Chaimbault *et al.*, 1999) detectors.

Table 3 Examples of extraction, clean-up, separation and detection methods applied in the latest fifteen years for the analysis of amino acids in different matrices.

Matrix	Particulate Matter	Extraction	Clean-up	HPLC		Detector and Detected Amino Acids	Reference
				Mobile Phase	Stationary Phase		
Aerosols	Bulk	Sonication (MeOH*)	Filtration	Eluent A: CH_3COONa aqueous buffer - pH 4.00 Eluent B: ACN and HCOOH	ZIC-HILIC	(+)ESI-MS/MS **20** A, N, D, C, Q, E, G, 3-Hyp, 4-Hyp, I, L, M, MetSO, $MetSO_2$, F, P, S, Y, T, V	(Barbaro *et al.*, 2011)
	$PM_{0.1}$ $PM_{2.5}$ PM_{10}	Sonication (H_2O/MeOH), evaporation, clean-up, hydrolysis and purification	Solid phase extraction	Eluent A: H_2O and HCOOH (pH 2.3) Eluent B: MeOH and HCOOH	C18	(+)ESI-MS/MS **24** Orn, H, K, R, G, A, S, P, V, T, L, I, N, Q, D, Hyp, E, Cit, C, M, F, Y, W, GABA	(Di Filippo *et al.*, 2014)
	PM_{10}	Sonication (MeOH)	Extracts assemblage and filtration	Eluent A: CH_3COONa aqueous buffer - pH 4.00 Eluent B: ACN and HCOOH	ZIC-HILIC	(+)ESI-MS/MS **20** F, T, L, I, M, Y, V, 3-Hyp, P, 4-Hyp, A, E, Q, S, N, D, C, G, $MetSO_2$	(Scalabrin *et al.*, 2012)
	Bulk	Sonication (pure H_2O)	Filtration	*Not specified*		**14** D, S, E, G, A, V, M, I, L, Y, F, H, K, R,	(Matsumoto and Uematsu, 2005)
	PM_{10}	Sonication (ultrapure H_2O and ice; addition of ice); spike and extraction (ultrapure H_2O)	Filtration	Eluent A: ultrapure H_2O and HCOOH Eluent B: MeOH and HCOOH	CHIROBIOTIOC TAG	**11** A, D, R, E, F, P, Y, T, L, G, V	(Barbaro *et al.*, 2014 - a))

Aerosols (from biomass burning)	PM_{10}	*For the analysis, filtered aerosol extracts were injected onto the column without further preparation*		ACN:MeOH and a pH-neutral potassium phosphate dibasic solution	Dionex DX 300	UV-Vis **17** D, E, S, T, G, A, R, P, V, M, I, L, F, C, K, H, Y	(Mace *et al.*, 2003)
Urban dust	PM_{10}	Accelerated solvent extraction (H_2O/MeOH)	Solid phase extraction, wash and elution	Eluent A: CH_3COONH_4, adjusted to pH 4.00 (HCOOH) Eluent B: ACN/H2O acidified (HCOOH)	C18, LUNA HILIC and Acclaim Trinity	MS/MS **14** F, P, L, I, M, V, W, Y, A, T, Q, G, S, N	(Buiarelli *et al.*, 2013)
Soils	Bulk	Dissolution (ultrapure H_2O) and centrifugation	---	CE*		MS/MS Only peptides: G-G, A-A, G-L,G-Q, G-E, G-H, A-A-A, G-Y, V-Y-V, Glutathione, Y-G-G-F-L, Y-G-G-F-M, D-R-V-Y-I-H-P-F, Cystathionine	(Warren, 2013)
	Bulk	Acid hydrolysis, addition of internal standard solution	Filtration, dryness and re-dissolution; polypropylene sample preparation column (cationic exchange resin)	Nitrogen (carrier gas)	GC*	MSD and FID **15** A, V, G, T, I, L, P, S, D, M, F, E, Y, Orn, K	(Amelung and Zhang, 2001)

Soils	Bulk	Addition of KCl, agitation and centrifugation	Filtration	CE*		LIF **17** R, K, L, I, F, V, Y, T, GABA, A, D, E, G, C, N, H, Q	(Warren, 2008)
	Bulk	Addition of H_2O, agitation, centrifugation, filtration, freeze dry; re-dissolution in H_2O	Filtration	*Not mentioned*		Fluorescence **15** H, G, T, D, E, S, A, R, Y, C, V, M, F, I, L	(Johnson and Pregitzer, 2007)
	Bulk	Acid hydrolysis, filtration, drying and re-dissolution	Polypropylene column with a cationic exchange resin, re-dissolution and centrifugation	Eluent A: NH_4HCO_2 in H_2O, adjusted to pH 3.0 (HCOOH) Eluent B: ACN Eluent C: H_2O	C18	(+)ESI-MS/MS **17** F, L, I, V, M, Y, K, C-C, P, A, T, E, D, G, R, S, H	(Hou *et al.*, 2009)
	Bulk	Dissolution in H_2O	Filtration	*Not mentioned*		Fluorescence **18** A, R, N, D, E, Q, G, H, I, L, K, M, F, S, T, W, Y, V	(Werdin-Pfisterer et al., 2009)
	Bulk	Dissolution in H_2O	Filtration	*Not mentioned*		**18** A, R, N, D, E, Q, G, H, I, L, K, M, F, S, T, W, Y, V	(Werdin-Pfisterer et al., 2012)

Barley	Bulk	Sample grinding in liquid nitrogen (mortar and pestle); addition of an aqueous HCl-C_2H_5OH solution; centrifugation	---	Eluent A: CH_3COONa, adjusted to pH 6.00 (CH_3COOH) Eluent B: CH_3COOH	Phenomenex Luna	(+)ESI-MS/MS **20** R, K, H, C-C, W, I, L, F, T, V, M, A, P, G, Y, Q, N, S, E, D	(Thiele *et al.*, 2008)
Mural Painting	Bulk	Grind and hydrolysis, oil bath under a nitrogen atmosphere, dilution (ACN)	Filtration	Eluent A: CH_3COONa, adjusted to pH 4.00 (HCOOH) Eluent B: ACN acidified (HCOOH)	ZIC-HILIC	(+)ESI-MS/MS **12** F, T, L, I, V, P, A, 3-Hyp, 4-Hyp, E, S, D	(Zangrando *et al*., 2010)
SRM	Bulk	---	---	Eluent A: PDFOA in H_2O and TDFHA in H_2O Eluent B: ACN	Purospher RP-18e and Supelcosil ABZ Plus	LC-API-MS And LC-ELSD **17** D, N, S, G, Q, C, E, T, A, P, V, M, Y, I, L, F, W	(Chaimbault *et al.*, 1999)
Water	---	Bottle 1: filtration, freezing and spike. Bottle 2: freezing, defrost, sonication and spike		Eluent A: HCOOH Eluent B: MeOH containing HCOOH	CHIROBIOTIC TAG	MS/MS **21** A, R, D, N, Q, E, G, Hyp, H, L, I, K, M, Orn, F, P, S, T, W, Y, V	(Barbaro *et al.*, 2014 - b))

MeOH – methanol; ACN – acetonitrile; MS – mass spectrometry; SRM – standard reference material; PDFOA – pentadecafluorooctanoic acid; TDFHA – tridecafluoroheptanoic acid; CE – capillary electrophoresis; GC – gas chromatography; MSD – mass selective detector; FID – flame ionization detector; LIF – laser-induced fluorescence; GABA – gamma-aminobutyric acid

As shown in **Table 3**, the three most used columns for amino acids separation are ZIC-HILIC, C18 and CHIROBIOTIC TAG. Barbaro *et al.* (2011), Scalabrin *et al.* (2012) and Zangrando *et al.* (2010) chose to separate the analytes with a ZIC-HILIC column, in order to distinguish them according to their hydrophobicity. Although they used the same stationary phase, they studied different matrices: aerosols (Barbaro *et al.*, 2011; Scalabrin *et al.*, 2012) and mural painting (Zangrando *et al.*, 2010). Trying to achieve the same goal, i.e., separation of amino acids according to their tendency to interact with H_2O, Di Filippo *et al.* (2014) and Hou *et al.* (2009) used a C18 stationary phase. Barbaro *et al.* (2014a, 2014b) aimed at separating the *D*- and the *L*- enantiomers from the same amino acid (alanine, arginine, asparagine, aspartic acid, glutamate, histidine, leucine, isoleucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, hydroxyproline and ornithine), and therefore the authors used the CHIROBIOTIC TAG column.

Concerning the chiral separation of amino acids, there are two methods that can be employed. One is a direct method based on the formation of diastereomers on the stationary or mobile phases. However, resolving the enantiomers on polysaccharide-based **chiral stationary phases (CSPs)** can be a difficult task, since underivatized amino acids are zwitterionic (neutral molecule with opposites charges in different atoms) and poorly soluble in non-polar solvents, such as hydrocarbons (Haginaka, 2013). The second method is an indirect method, based on the formation of diastereomers via the reaction of amino acids with a chiral derivatization reagent and then separation on an achiral stationary phase. Derivatization before separation can improve solubility or create diastereomers capable of being resolved by an achiral column. Nevertheless, as shown by Ilisz *et al.* (2008), this method adds an extra step and possible impurities to the sample.

2. Liquid Chromatography: basic concepts

2.1 - Definition and brief history

The possibility of having numerous stationary-mobile phases combinations regarding chromatography that can be used to separate a mixture leads to the existence of different types of chromatography, classified according to the physical state of those phases. In the liquid chromatography technique, it is used a liquid mobile phase which that passes through a solid stationary phase along with the sample. The liquid chromatography technique is applied to separate the several compounds that form a particular sample. The separation is based on the interactions of the components of the sample with the mobile and the stationary phases (Skoog *et al.*, 2007).

Liquid chromatography was first introduced in the beginning of the XX century, in 1901, by the Russian Mikhail Tswett, who separated leaf pigments from plants using a solvent in a column packed with particles – calcium carbonate and alumina (Arsenault and McDonald, 2014).

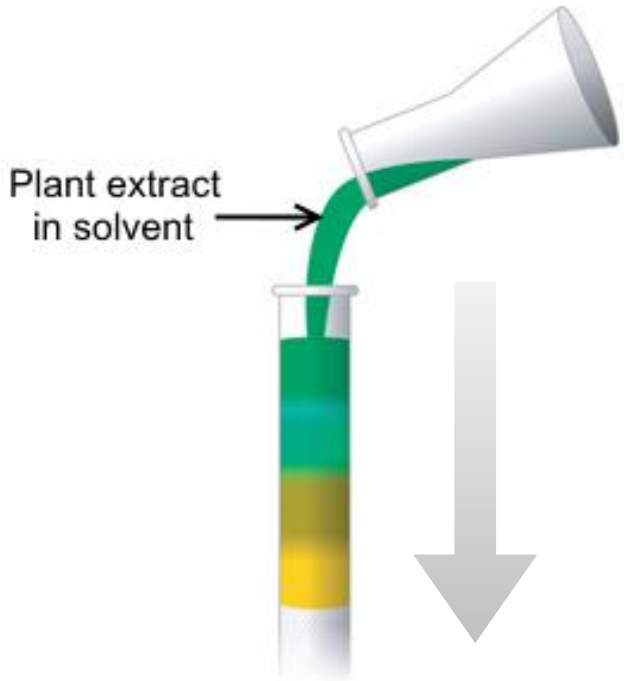


Figure 5 Tswett's experiment that represented the inception of liquid chromatography (adapted from Arsenault and McDonald, 2014).

The botanist filled an open glass column with calcium carbonate (powdered chalk) and alumina and poured the sample (solvent extract of homogenized plant leaves) into the column, allowing it to pass into the particle bed. As the sample was going down through the column by the gravity force, different colored bands appeared in the column, as shown in **Figure 5**. With this result, the botanist related the distinct marks to the different components contained in the sample, realizing some compounds were moving faster than others during the separation, which caused the separated, different-colored bands (Arsenault and McDonald, 2014).

In 1952, Archer John Porter and Richard Laurence Millington Synge were awarded the Nobel Prize due to the establishment of the basics of partition chromatography and the development of the Plate theory, which represented a relevant growth in the chromatography technique. Nowadays, liquid chromatography has become one of the most powerful separation methods in analytical chemistry (Skoog *et al.*, 2007).

2.2 - The development of the HPLC technique

Initially, liquid chromatography was performed in glass columns with 10 to 50 mm of diameter and they were packed with 50 to 500 cm lengths of solid particles coated with an adsorbed liquid that made the stationary phase. In order to guarantee reasonable flow rates in this type of stationary phase, the particle size of the solid components were kept larger than 150 μm and smaller than 200 μm. However, the flow rates were, maximum, a few tenths of a milliliter per minute, which caused the separation times to be extremely long – sometimes, the analysis lasted several hours. Although there were attempts to increase the quickness of this procedure with the application of pressure or vacuum, they were not effective, which can be explained by the relation between the flow rates and the plate heights explicit in the Plate Theory: there is an ideal value for the flow rate and the plate height to achieve the highest column efficiency possible during the analysis. That value is specific of each column, as shown in **Figure 6**, and can be obtained using the Van Deemter equation (1). In chromatography, a theoretical plate is a hypothetical zone in which the two phases, establish an equilibrium; the plate height is the height equivalent to a theoretical plate in the column (Yip, 1997).

$$H = A + \frac{B}{\mu} + C * \mu \qquad (1)$$

H – Height of the theoretical plate [*m*]

A – Eddy diffusion (multi-path effect: not all the molecules of the mobile phase are eluted at the same speed through the stationary phase) [*m*]

B – Longitudinal diffusion [$m^2.s^{-1}$]

C – Mass transfer (effect caused by the adsorption and absorption of the particles from the mobile phase within the stationary phase) [*s*]

μ – Linear velocity of the mobile phase [$m.s^{-1}$]

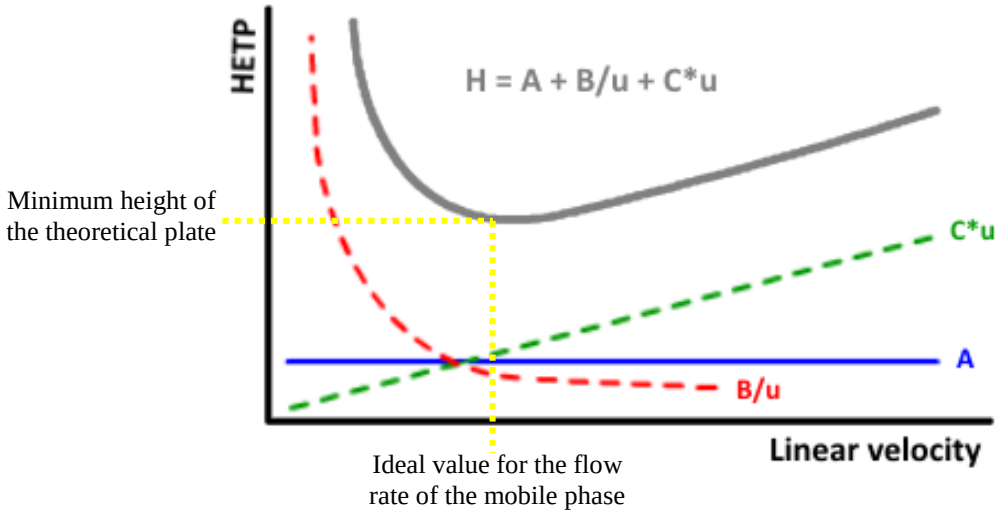


Figure 6 Graphical representation of the contributing terms for the band broadening (adapted from Yip, 1997).

In 1956, Van Deemter derived an equation that included the factors that contribute the most to band broadening in the chromatogram – phenomenon that decreases the efficiency of the separation process, leading to a poor resolution. Although the band broadening increases with the use of the chromatographic column, there are some actions that can be taken to reduce this process, in order to ensure maximum efficiency of the column and minimum band broadening (Yip, 1997).

As it is possible to observe in **Figure 6**, a growth of the flow rate tends to increase the plate height above its' minimum ideal value, leading to a decreased of the efficiency of the column. Thus, trying to develop the liquid chromatography technique by

controlling other variables different from the flow rate, scientists realized that major increases in column efficiency could be achieved by decreasing the particle size of packings. Only around 1960 the technology to produce and use packings with particle diameter from 3 to 10 µm was developed, requering sophisticated instruments operating at high pressures, in contrast with the simple glass columns of classic gravity-flow liquid chromatography. This marked the invention of the High Performande Liquid Chromatography (HPLC). Nowadays, all liquid chromatography techniques are performed with pressurized flow and the acronym HPLC is used interchangeably. HPLC is one of the most used of all the analytical separation techniques due to its: sensitivity, ease of automation, suitability for separating nonvolatile species or thermally fragile ones, ready adaptability to accurate quantitative determinations and widespread applicability to substances that are strongly important to industry and many fields of Science, such as amino acids, proteins, nucleic acids, drugs, hydrocarbons, terpenoids, pesticides, carbohydrates, steroids, metal-organic species, antibiotics and a diversity of inorganic substances (Scott, 1995).

2.3 - Separation mechanisms in liquid chromatography

As aforementioned, in the liquid chromatography analysis, as the mobile phase goes through the column, the analytes interact with the solid stationary phase. The analytes in the mobile phase are separated based on those interactions, which depend on the physicochemical properties of the analytes, the mobile and the stationary phase. According to the nature of the interaction between the analyte and the stationary phase, the chromatography separation mechanism can be classified as **adsorption**, **partition**, **ion exchange**, **size-exclusion** or **affinity**. Besides the individual use of the separation mechanisms, it is possible to assemble them, and consequently, apply a **mixed-mode** (Skoog *et al.*, 2007). The adsorption separation mechanism occurs when the solute is adsorbed onto the surface of the stationary solid phase. The separation is due to the different adsorptive properties of the different compounds of the sample. The most polar compounds have a bigger tendency to adsorb to the most polar phase while non-polar compounds adsorb to the non--polar phase. Hence, when it is used a stationary phase with

higher polarity than the mobile phase, the most polar components elute later than the non-polar ones, since their interaction with the solid phase is longer. The contrary happens when the liquid mobile phase is more polar than the stationary phase: the non-polar components are eluted later than the components with bigger polarity (Skoog *et al.*, 2007). The partition separation mechanism is based on a thin film formed by a liquid stationary phase on the surface of a solid support. In this type of mechanism, both the stationary and the mobile phases must be liquid (immiscible) or gas (liquid-liquid, liquid-gas or gas-gas), since the partition is a phenomenon that can not occur in a solid environment. Partition is a process of separation whereby the components of the sample get distributed into two solvents. The molecules get dispersed into the phases and the most polar compounds get partitioned into the most polar phase (Skoog *et al.*, 2007). When the solutes being separated are ionizable molecules and the separation is performed based on their total charge, the separation mechanism is called ion exchange. This method allows the separation of molecules with similar properties that would be difficult to separate by other techniques because, in this case, the charge of the analyte can be manipulated by changing the pH of the mobile phase. The stationary phase is made of a solid resin in which the ions of the solute are covalently attached onto. The ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces: an anionic resin attracts cations and the anions on the mobile phase are attached to a cationic stationary phase (Skoog *et al.*, 2007). Size-exclusion separation mechanism is a method that separates the particles in the mobile phase according to their molecular size. This process separates the analytes by trapping the smaller molecules in the pores of the stationary phase (a porous gel), while the larger molecules do not enter the pores during the elution. Thus, larger molecules flow through the column faster than smaller molecules, sorting the molecules by their size. In this method, the smaller the molecule, the bigger the retention time (Skoog *et al.*, 2007). The affinity mechanism is the most selective type of separation as it utilizes the specific interaction between one kind of molecule in the solute and a second molecule that is immobilized on the stationary phase. After the binding, only the molecules that match this precise property are retained in the column, while the unbound compounds flow through the column in the mobile phase. This separation mechanism is often used to purify proteins: the immobilized molecule is an antibody to a specific protein; the solute containing a mixture of proteins is passed by this molecule and only the specific protein reacts with the antibody, binding the new molecule to the stationary phase. After, the protein can be extracted by changing the ionic strength or the

pH, in order to elute the protein and have it purified (Skoog *et al.*, 2007). Mixed-mode is based on columns that have been functionalized with ligands capable of multiple modes of interaction: ion exchange, hydrophilicity and size-exclusion, for example. The ability to combine these separation methods can boost the selectivity in the separation process. The different interactions are not independent of one another. For example, when using a mixed-mode ligand containing both hydrophobic and ionic elements, the increase of the ionic strength will disrupt ionic bonds. However, the increase of salt concentration will promote the hydrophobic interactions (Skoog *et al.*, 2007).

3. Final considerations and proposal for thesis work

Concerning the separation of amino acids from the remainder organic matter in bioaerosols samples, liquid chromatography has the potential to be an accurate technique. Therefore, the final proposal for the separation method regarding the main goal of this work includes the procedure schematically represented in **Figure 7**.

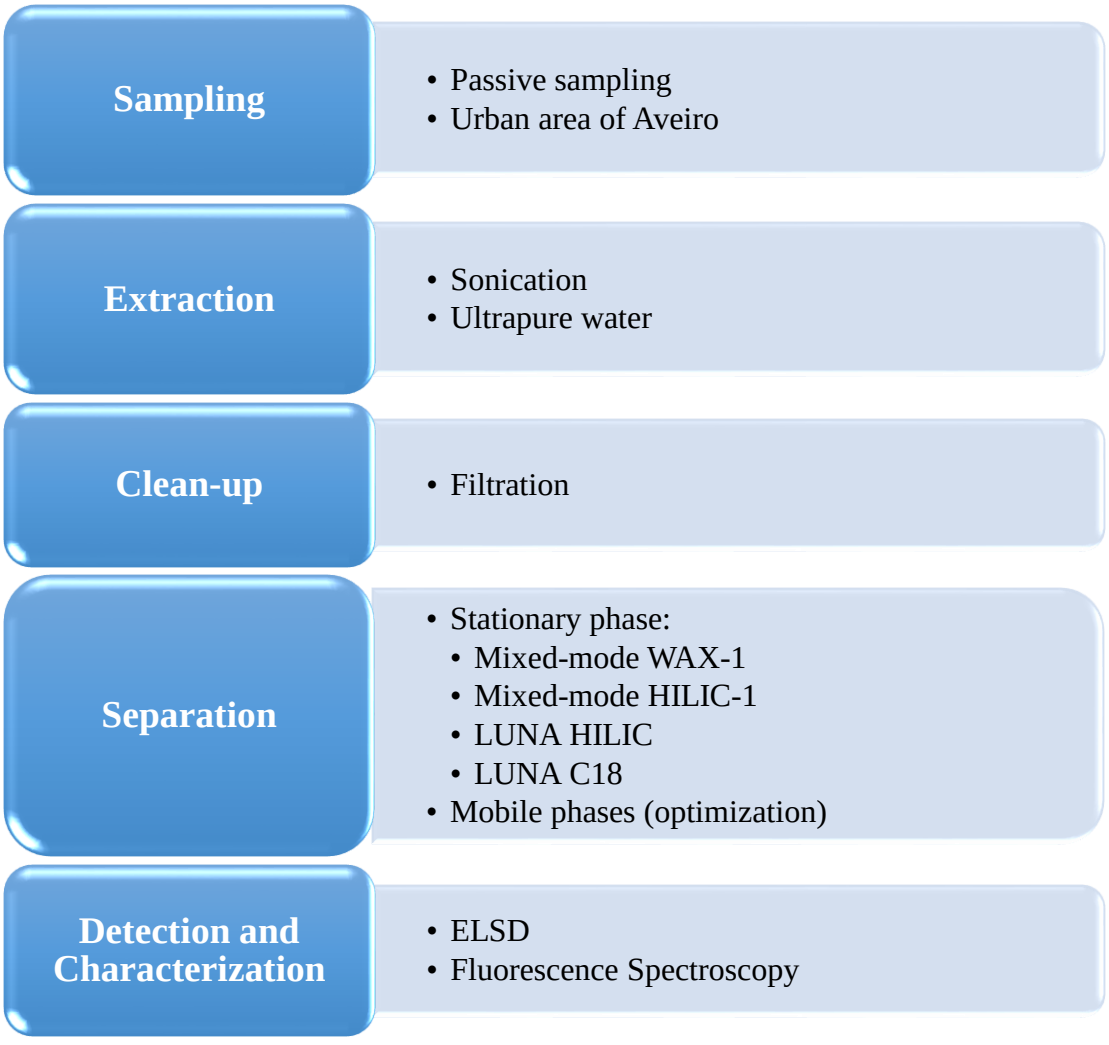


Figure 7 Proposal for the analytical procedure to be employed in this work.

Since it preserves the representativeness related to the natural process of deposition of the sample, it will be used a passive sampler, designed purposely for this study, in the urban area of Aveiro. It is relevant to note that the sampling of the bioaerosols

will be dependent on the weather conditions. After the collection, the samples will be treated via sonication in ultrapure water. In order to clean-up the organic phase, the resulted extract from the sonication will be cleaned by a filtration process, as done by Barbaro *et al.* (2014a). After that, the solution will be analyzed by a liquid chromatography method, enabling the separation of the amino acids present in the collected bioaerosols. In order to select the best column to separate amino acids in a bioaerosols sample, several columns will be used in the preliminary tests with standard amino acids: Mixed-Mode WAX-1, with WAX (**weak anionic exchange**), HILIC (**hydrophilic interaction chromatography**) and RP (**reversed phase**) functionalities; Mixed-Mode HILIC-1, with HILIC and RP capabilities; HILIC; and C18, with RP functionalities. Next, and according to the results, the separation mechanism that allows the best separation of the amino acids during the preliminary analyzes will be used to separate the amino acids in the collected samples of bioaerosols. Although the composition of the mobile phases has to be optimized according to each stationary phase, it is expected the use of the following mixtures of eluents, following the procedure of Barbaro *et al.* (2014a): ultrapure water with formic acid and MeOH with formic acid.

As the final step, the analytes will be detected via ELSD and characterized via fluorescence spectroscopy. The ELSD is the detector of choice due to the adequate limits of detection regarding amino acids and the fact that it does not require the presence of a chromophore group in the analytes. The ELSD is a destructive technique, therefore, the samples can not be utilized again after the ELSD detection (Petritis *et al.*, 2002).

II

Materials and methods used for the study of amino acids separation

1. Liquid chromatography columns

1.1 - Acclaim® Mixed-Mode WAX-1

The packing of this column consists in a silica gel-based stationary phase that incorporates both weak anion-exchange and hydrophobic properties. As shown in **Figure 8**, the packing material has a hydrophobic alkyl chain with an ionizable terminus allowing the chromatographer to have total control of selectivity in the separation of acids, bases and neutral molecules, unlike the traditional RP columns (Phenomenex Columns Manual – a)).

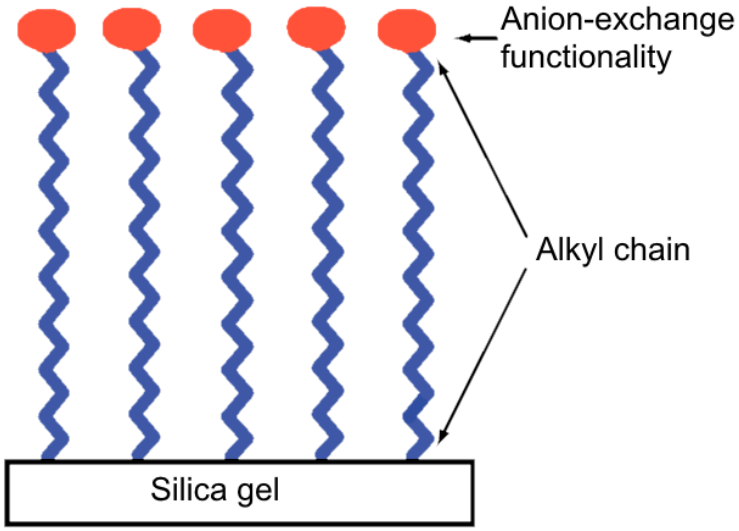


Figure 8 Surface composition of the Acclaim® Mixed-Mode WAX-1 column (adapted from Phenomenex Columns Manual - a)).

The Acclaim® Mixed-Mode WAX-1 is a column that provides: selectivity complementary to RP columns (more than one separation mode allows different elutions orders); adjustable selectivity (possibility of controlling the elution by changing the chromatographic conditions); simultaneous separation of acidic, basic and neutral molecules due to the various operating modes; high capacity and unique selectivity for anionic molecules (hydrophobic retention alone is not adequate to differentiate molecules with similar values of hydropathy); and the opportunity to operate with the following

multimode retention mechanisms - RP, anion-exchange and HILIC modes (Phenomenex Columns Manual – a)).

The fact that this column combines all the mentioned modes facilitates the selectivity adjustment by changing the mobile phase ionic strength, pH or organic content, one at a time or all at the same time. An increase of the ionic strength causes a retention time: decrease, in acids; increase, in case of bases; and no effect for neutral molecules. In the case of the hydrophobic retention, the retention time is affected by the organic content of the mobile phase. Generally, all types of molecules are less retained when the organic content of the mobile phase increases and the other conditions are kept constant. Although pH has no relevant effect on the retention of neutral molecules, it affects significantly anionic molecules: decreasing the pH reduces the negative charge of molecules containing carboxylic groups, leading to a decrease of the retention. Considering the effect that changing the pH can have in the retention results and, moreover, in order to not damage the stationary phase, the Acclaim® Mixed-Mode WAX-1 column should not be used without a proper buffer in the mobile phase. In this column, the HILIC mode is predominant over the WAX and RP modes when the organic solvent represents more than 70% (V/V) of the mobile phase. When in the HILIC mode, highly polar molecules retention increases (Phenomenex Columns Manual – a)).

1.2 - Acclaim® Mixed-Mode HILIC-1

The Acclaim® Mixed-Mode HILIC-1 column is, like the Acclaim® Mixed-Mode WAX-1, formed by a spherical silica-based mixed-mode stationary phase that integrates both RP and HILIC properties. As it is possible to observe in **Figure 9**, the column packing incorporates a long-chain alkyl group with a hydrophilic polar terminus (diol group), which allows the separation of a wide range of polar and non-polar molecules (Phenomenex Columns Manual – b)).

The use of the Acclaim® Mixed-Mode HILIC-1 column provides several advantages such as: operate in both RP and HILIC modes; retention of highly polar molecules that are not retained by traditional RP columns; selectivity complementary to RP columns; and a superior performance compared to traditional diol-based columns.

Conventional HILIC stationary phases (for example, unmodified silica, amino, diol phases) have hydrophilic surfaces, which does not allow the separation of small molecules via hydrophobic interaction. Thus, a column with both functionalities provides superior chromatographic properties and supports a broader variety of applications. Regarding its' modes, the column works in RP mode when the mobile phase contains less than 75% (V/V) of the organic solvent. In this mode, the retention time of a hydrophobic (non-polar) compound increases as the organic solvent content of the mobile phase decreases. On the other hand, in order to ensure the column is functioning in the HILIC mode, the mobile phase must contain more than 90% (V/V) of the organic solvent. In this mode, the retention of hydrophilic oligomers increase as the mobile phase content in organic solvent also increases. To control the elution of the samples, the organic solvent content is the crucial condition that must vary in this column (Phenomenex Columns Manual – b)).

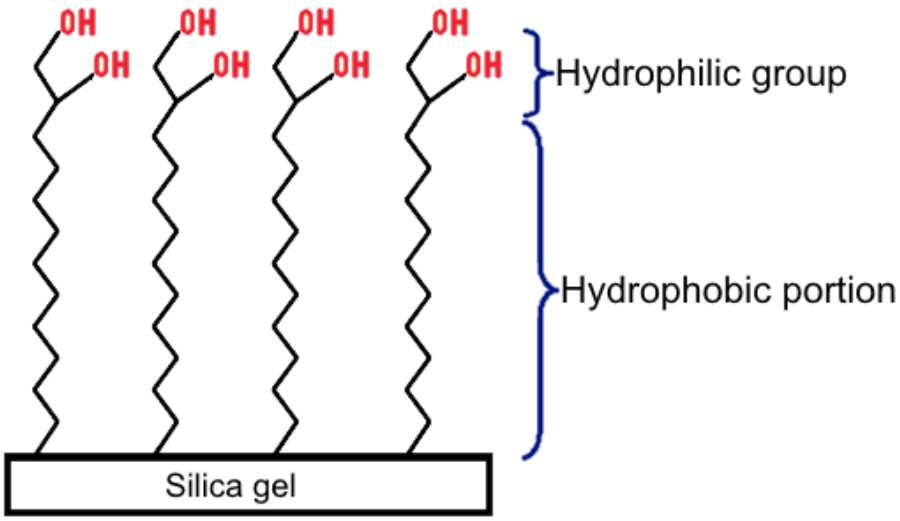


Figure 9 Surface chemical composition of the Acclaim® Mixed-Mode HILIC-1 column (adapted from Phenomenex Columns Manual - b)).

1.3 - Phenomenex® Luna HILIC

The Phenomenex® Luna HILIC column has a silica surface that is covered with cross-linked diol groups, as shown in **Figure 10**, for polar selectivity under hydrophilic conditions. The diol groups phase provides dipole-dipole interactions, non pH dependent hydrogen bonding, retention of the water layer that attracts the polar compounds, and the nonexistence of dissociable moieties. This column works only on the HILIC mode, has a wide pH stability (1.8 – 8.0) and it can be used for several purposes, from preparative chromatography to bulk purification. Luna HILIC column allows the increase of polar compounds retention in a mobile phase with a high organic solvent content. The functionalized silica surface draws and retains water on the its' surface. The motionless segment water-enriched layer facilitates the transfer of polar compounds into the stationary phase, where the retention of the analytes occurs. Under HILIC conditions, the polar compounds will interact strongly with this polar stationary phase, which leads to an increase of their retention time. This represents a meaningful benefit since the Luna HILIC column will retain the polar compounds that elute near the void in the RP mode (Phenomenex Columns Manual – c)).

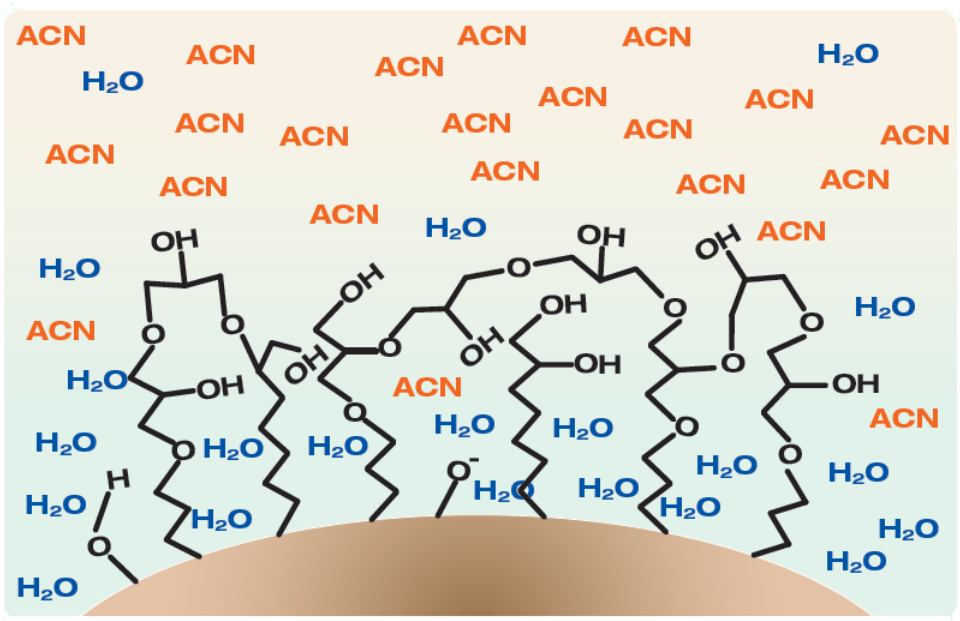


Figure 10 Surface composition of the Phenomenex® Luna HILIC (adapted from Phenomenex Columns Manual - c)).

1.4 - Phenomenex® Luna C18

In this column, octadecyl silane ligands are bound to the silica surface, creating a hydrophobic phase, as shown in **Figure 11**, and it operates under RP conditions. The column has a wide pH stability (1.5 – 10) due to the high ligand surface density that features its' surface, and a method flexibility. Moreover, this column is meticulously made in order to guarantee the surface smoothness, pore structure and pore consistency to ensure particles have a uniform structure and enhance the mechanical strength of the column. The particular smoothness and sphericity of Luna C18 columns provides a uniform bonding surface for the phase coverage since the particles pack more easily. The probability of silica particle brake during bonding and packing is very low, which increases the efficiency and the lifetime of the column. In comparison with C18 columns from other brands, Phenomenex® Luna C18 column represents a measurable improvement in two crucial chromatographic properties: resolution and peak shape. The high efficiency and the bonded phase surface coverage of the column lead to sharp peaks, which results in the good method sensibility and the pH stability in long term use (Phenomenex Columns Manual – d)).

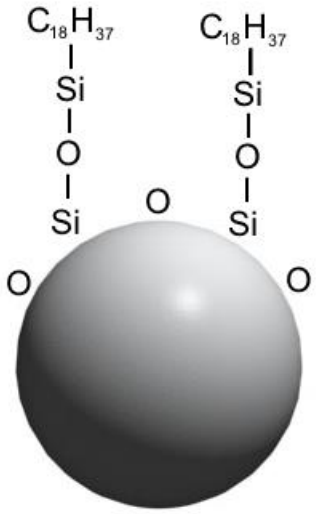


Figure 11 Surface chemical composition of the Phenomenex® Luna C18 (adapted from Phenomenex Columns Manual - d)).

2. Chemicals

All chemicals used in this work were of analytical reagent grade and obtained from commercial suppliers without further purification. The solutions were all prepared with ultra-pure water (18 M Ω cm) and the mobile phases for the liquid chromatography analyzes were prepared with HPLC grade MeOH, acetonitrile (CH_3CN) and ammonium acetate (CH_3COONH_4) buffered with acetic acid (CH_3COOH). The standard individual amino acids were purchased from Sigma-Aldrich.

3. Preparation of the solutions for the preliminary tests

The compositions of the mobile phases were adjusted according to the column in use and they are described in the section **III – Preliminary tests**. All the solvents of the mobile phases were filtered through membrane filters (Durapore®, Gelman Sciences) of 0.22 μm pore size. After the filtration, the solutions were taken to the sonication for 15 minutes, except when the Phenomenex® Luna C18 column was used. There was a change in the equipment from the first three columns (Acclaim® Mixed-Mode WAX-1, Acclaim® Mixed-Mode HILIC-1 and Phenomenex® Luna HILIC) to the fourth one (Phenomenex® Luna C18), and the pump coupled to the Phenomenex® Luna C18 column had its own degasser system, erasing that step in the preparation of the mobile phase.

The standard solutions were prepared by the dissolution of the individual amino acids compounds in the same solvents that constituted the respective mobile phase: ultra-pure water, buffer, MeOH and acetonitrile. The individual amino acids standard solutions had a concentration of 1 mM.

4. Passive sampling

4.1 - Passive sampler

To collect atmospheric aerosol samples in the urban city of Aveiro, a passive sampler was built. Its design is shown in **Figure 12** and it was made for the holder to be embedded in the weather station available in the laboratory.

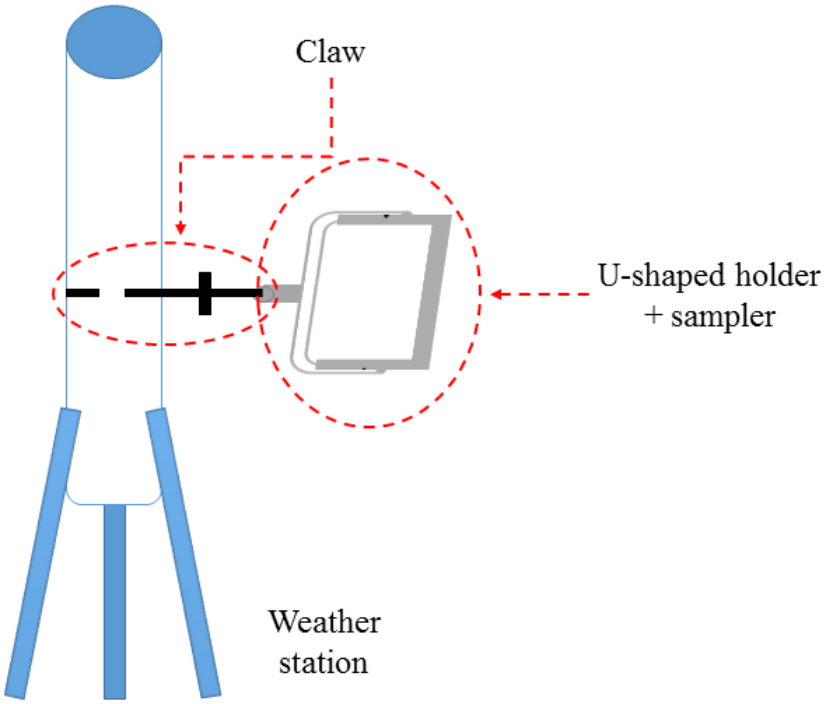


Figure 12 Representation of the sampler and its holder embedded in the weather station.

The sampler was constituted by three different parts, as it is possible to observe in **Figure 13**: the sampler support made of steel; two polyvinyl chloride plates; and the sampler itself, a plate made of Teflon, where the filters were put, placed between the two polyvinyl chloride plates. Each sampler had two quartz microfiber filters (Whatman™, 20.3 × 25.4 cm): one on top of the Teflon plate and the other on the bottom of it. The filters were placed between the Teflon and the polyvinyl chloride plates. The weight of each filter was measured before and after the sampling and those values can be found in the **Table 4**.

Figure 13 Picture of one model of the samplers. It is possible to distinguish the three parts that constitute the sampler: the support; the two polyvinyl chloride frames (grey plates); and the Teflon plate where the filters were placed (white plate).

The sampling occurred during one month for each sampler, in two distinct spots in Aveiro: one near the Glicínias Plaza mall, in front of a construction site (**Figures 14 to 16**); and two at the roof of the STIC Department of the University of Aveiro (**Figures 17 to 19**). The first mentioned sampler was installed on 15th June around 19h 25min and it was collected on 16th July around 11h. In the University of Aveiro, two samplers were installed on 18th June around 16h 30min and they were collected on 20th July around 11h. One of these samplers was put on the horizontal while the other was on the vertical position. In this last case, the filters were not placed up and bottom but left and right, regarding the position of the weather station.

From 10h 30min of 22nd June until 11h of 23rd June, the filters were protected with aluminum foils and plastic bags in order to protect them from the rain. The same happened from 18h of 30th June until 10h 30min of 2nd July.

Figure 14 Front view of the sampler installed near the Glicínias Plaza mall.

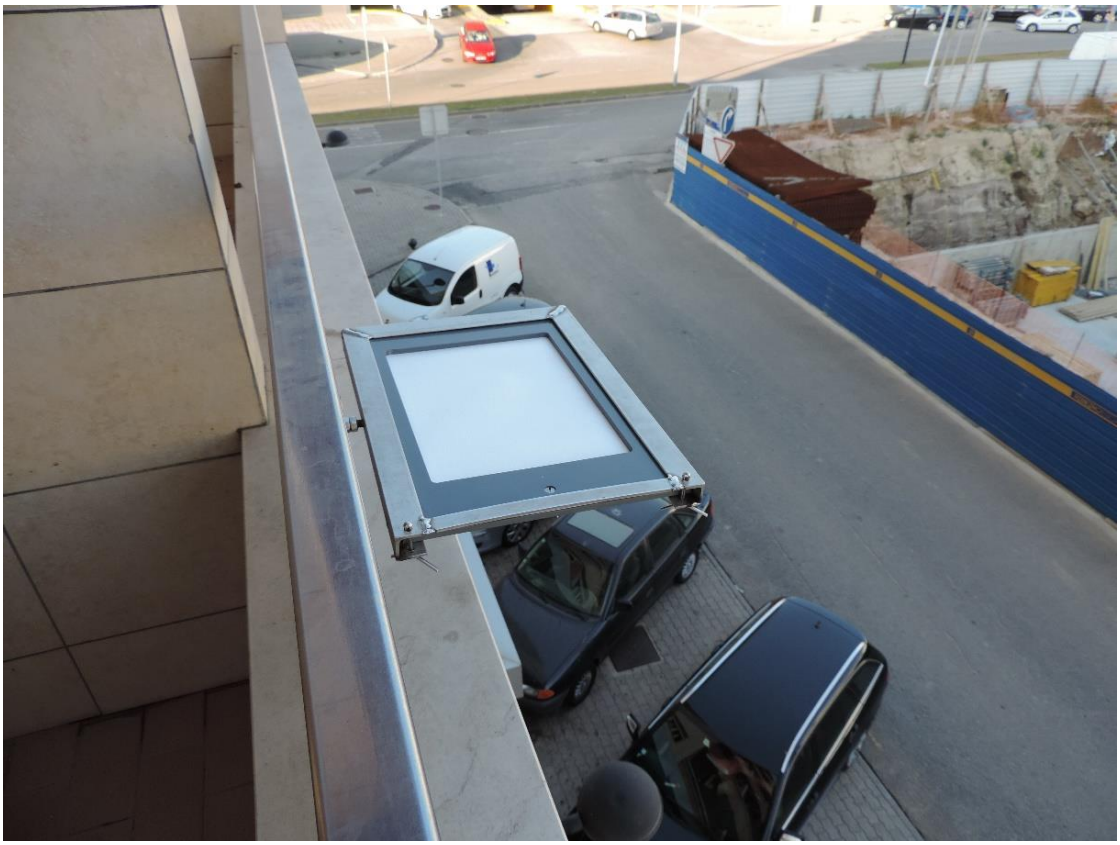


Figure 15 Lateral view of the sampler installed near the Glicínias Plaza mall.

Figure 16 Building where the sampler was installed.

Figure 17 Surrounding of the samplers on the roof of the STIC Department at the University of Aveiro.

Figure 18 Surrounding of the samplers on the roof of the STIC Department at the University of Aveiro.

Figure 19 Weather station with the two samplers in the roof of the STIC Department at the University of Aveiro.

The up filter of the sampler near Glicínias Plaza mall had visible biologic matter, as shown in **Figure 20**, possibly due to birds activity.

Figure 20 Up filter of the sampler installed near the Glicínias Plaza mall with biologic matter.

Table 4 Weight of the filters used during the sampling. The sample weight was calculated by the difference of the mean value of the after and the before sampling measure.

Filter	Sampler near Glicínias Plaza mall				Sampler at the STIC Department - horizontal				Sampler at the STIC Department - vertical			
	Before sampling		After sampling		Before sampling		After sampling		Before sampling		After sampling	
	Bottom	Up	Bottom	Up	Bottom	Up	Bottom	Up	Bottom	Up	Bottom	Up
Weight (± 0.0001 g)	4.3242	4.3233	4.3305	4.8359	4.3268	4.3315	4.3304	4.3747	4.3313	4.3197	4.3369	4.3321
	4.3243	4.3230	4.3290	4.8361	4.3258	4.3315	4.3302	4.3746	4.3308	4.3194	4.3366	4.3321
	4.3245	4.3215	4.3292	4.8360	4.3259	4.3315	4.3304	4.3748	4.3306	4.3196	4.3366	4.3319
	4.3243	4.3218	4.3289	4.8361	4.3259	4.3316	4.3304	4.3748	4.3305	4.3198	4.3365	4.3318
	4.3245	4.3226	4.3290	4.8361	4.3260	4.3316	4.3304	4.3749	4.3311	4.3196	4.3368	4.3321
Mean (± 0.0001 g)	4.3244	4.3224	4.3293	4,8360	4.3261	4.3315	4.3304	4.3748	4.3309	4.3196	4.3367	4.3320
Sample weight (± 0.0001 g)	Bottom		0,0049		Bottom		0,0043		Bottom		0,0058	
	Up		0,5136*		Up		0,0433		Up		0,0124	

* Filter in which the bird activity happened, as shown in **Figure 20.**

4.2 - Sampling data

During the sampling days, the atmospheric conditions were registered from the Associated Laboratory CESAM real-time online database (Current Weather in Aveiro, Portugal) every 12 hours, as shown in **Table 5**. The zero hour represents the 15th June, 19h 30min.

Table 5 Atmospheric conditions in Aveiro during the sampling time (data collected from Current Weather in Aveiro, Portugal). The values shading in light grey refer to the evening moments while the others were registered in the morning.

Hour	Temperature (°C)	Humidity (%)	Dew point (°C)	Wind direction	Wind velocity (m/s)
0	17.8	84	15.1	NW	5.4
12	17.2	88	15.2	SSE	0.9
24	19.8	79	16.0	NNW	3.1
36	19.4	83	16.4	S	0.4
48	23.2	64	16.0	N	2.2
60	22.8	44	9.9	ENE	5.8
72	23.5	63	16.0	NW	3.1
84	25.8	43	12.2	NE	4.9
96	24.7	55	15.5	NW	2.8
108	27.3	37	11.3	ENE	2.2
120	28.8	46	16.0	NNW	1.8
132	22.6	73	17.5	SSE	1.3
144	20.3	87	18.1	SSW	1.3
156	17.8	93	16.7	S	3.6
168	18.2	80	14.7	S	1.8
180	17.7	79	14.0	S	0.9
192	17.7	78	13.8	NW	1.3
204	16.9	89	15.1	NNW	0.0
216	18.1	86	15.7	NNW	3.1
228	18.1	86	15.7	NW	0.0
240	17.2	89	15.4	NW	1.3
252	16.1	94	15.1	NNE	1.8
264	18.1	92	16.7	NNW	1.8
276	22.1	76	17.6	NW	3.6
288	18.9	89	17.1	N	4.5
300	17.3	90	15.6	NW	5.4
312	19.1	87	16.9	NW	3.6
324	16.4	98	16.1	S	0.0
336	19.1	93	18.0	W	0.0
348	16.3	98	16.0	SSW	1.8
360	18.7	88	16.6	SW	1.3

372	19.9	76	15.5	SE	1.3
384	18.7	88	16.7	NNW	3.6
396	18.4	80	14.9	N	2.2
408	18.3	87	16.1	NW	1.8
420	16.4	98	16.1	SE	0.0
432	19.6	83	16.6	SW	1.8
444	21.0	87	18.8	NW	7.2
456	19.0	84	16.2	NNW	5.4
468	18.3	78	14.4	NW	6.7
480	17.6	87	15.4	NNW	3.1
492	17.0	88	15.0	SSW	1.8
504	17.8	80	16.3	NNW	2.9
516	16.7	96	16.1	N	2.2
528	18.1	94	17.1	NNW	2.7
540	16.8	97	16.4	W	1.3
552	18.6	83	15.6	SSW	2.2
564	20.5	86	18.1	S	0.9
576	18.6	87	16.3	S	1.3
588	18.8	86	16.4	WNW	2.2
600	18.1	88	16.1	WNW	2.9
612	19.3	87	17.1	WNW	4.0
624	17.5	90	15.8	WNW	4.5
636	18.2	91	16.7	W	2.2
648	17.2	89	15.3	WNW	4.0
660	17.3	93	16.1	SSW	2.2
672	17.8	91	16.3	W	1.8
684	17.6	96	17.0	SSE	0.4
696	17.7	93	16.5	SSW	2.7
708	17.6	95	16.8	SSE	2.7
720	17.8	94	16.9	W	1.3
732	19.3	84	16.6	NNW	0.0
744	19.3	88	17.2	NW	1.8
756	18.1	98	17.8	WNW	2.2
768	18.1	96	17.5	N	3.1
780	19.3	92	17.9	SE	1.8
792	20.6	84	17.8	WNW	0.4
804	20.1	89	18.2	SSE	0.0
816	21.1	91	19.6	WNW	0.0
828	19.7	92	19.3	W	1.8
840	19.3	98	19.0	NNW	2.2
Mean	19.1	84	16.2	---	2.3

N – North; S – South; W – West; NE – Northeast; NW – Northwest; SE – Southeast; SW – Southwest; NNE – North-northeast; NNW - North-northwest; SSE - South-southeast; SSW - South-southwest; ENE - East-northeast; WNW - West-northwest.

5. Extraction and preparation of bioaerosols samples

The extraction procedure applied in this work was based on the one from Duarte *et al.* (2007). After the sampling, the filters were collected from the samplers, kept in aluminum foils and storage in the freezer until the extraction process, shown in **Figure 21**. Next to the filtration step, the resultant solution was put in a graduated cylinder in order to measure its' volume. The volume used in the extraction of each filter is shown in **Table 6**.

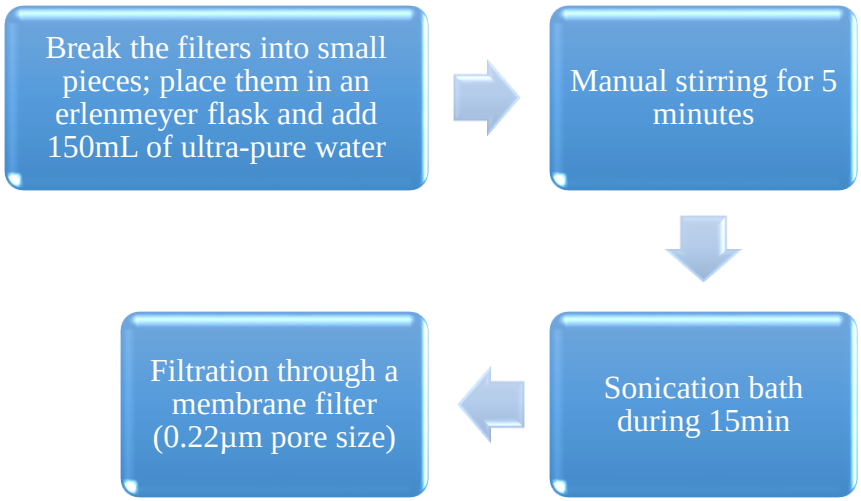


Figure 21 Procedure implemented for the extraction of the water-soluble organic compounds from the collected aerosols samples (adapted from Duarte *et al.*, 2007).

Table 6 Volume used during the extraction of the amino acids in each filter.

Filter	**Sampler near Glicínias Plaza mall**		**Sampler at the STIC Department - horizontal**		**Sampler at the STIC Department - vertical**	
	Bottom	Up	Bottom	Up	Bottom	Up
Volume (± 2.0 mL)	149	148	148	148	150	142

After the extraction process, the liquid samples were kept in glass flasks in the freezer. Posteriorly, the frozen samples were defrosted, transferred to round-bottom flasks, frozen in liquid nitrogen and, via lyophilization, the dry extracts of the collected samples were obtained. Before the freezing with liquid nitrogen, the weight of the each round-bottom flask was measured. After the lyophilization, those flasks' weight was measured again, and the values are shown in **Table 7**. The solid extracts were collected from the round-bottom flasks by dissolution on acidified water (pH=3.00), as shown in **Table 8**, and transferred to sample holder. The samples holder are shown in **Figure 22**.

Table 7 Volume used to dissolve the solid extracts obtained after the lyophilization step.

Filter	**Sampler near Glicínias Plaza mall**		**Sampler at the STIC Department - horizontal**		**Sampler at the STIC Department - vertical**	
	Bottom	Up	Bottom	Up	Bottom	Up
Volume	3.000	6.500	3.000	3.000	3.000	3.000

Table 8 Weight of the round-bottom flasks taken for the lyophilization step. The sample weight was calculated by the difference of the mean value of the after and the before lyophilization measure.

Filter	Sampler near Glicínias Plaza mall				Sampler at the STIC Department - horizontal				Sampler at the STIC Department - vertical			
	Before lyophilization		After lyophilization		Before lyophilization		After lyophilization		Before lyophilization		After lyophilization	
	Bottom	Up	Bottom	Up	Bottom	Up	Bottom	Up	Bottom	Up	Bottom	Up
Weight (± 0.0001 g)	109.9302	123.6639	109.9343	123.7546	124.0050	109.9946	124.0105	110.0099	100.1678	122.9533	100.1735	122.9593
	109.9301	123.6638	109.9342	123.7545	124.0050	109.9947	124.0105	110.0098	100.1677	122.9532	100.1734	122.9591
	109.9301	123.6638	109.9342	123.7546	124.0049	109.9946	124.0104	110.0099	100.1677	122.9532	100.1734	122.9590
	109.9301	123.6638	109.9342	123.7545	124.0048	109.9946	124.0104	110.0099	100.1677	122.9533	100.1733	122.9590
	109.9301	123.6637	109.9341	123.7545	124.0048	109.9947	124.0105	110.0099	100.1678	122.9533	100.1734	122.9590
Mean (± 0.0001 g)	109.9301	123.6638	109.9342	123.7545	124.0049	109.9946	124.0105	110.0099	100.1677	122.9533	100.1734	122.9591
Sample weight (± 0.0001 g)	Bottom		0,0041		Bottom		0,0056		Bottom		0,0057	
	Up		0,0907*		Up		0,0153		Up		0,0058	

* Sample collected from the filter in which the bird activity happened, as shown in **Figure 20**.

Figure 22 Vials containing the samples residues after the collection from the round-bottom flasks. The vial number 2 corresponds to the filter sample with biologic material from bird activity.

For the chromatographic analysis, 1.0 mL was collected from each sample, already dissolved in acidified water, filtrated through HPLC Certified Syringe Filters (hydrophilic regenerated cellulose of 0.20 µm pore size, SPARTAN, Whatman GmbH, Germany) and put in another sample holder (**Figure 23**).

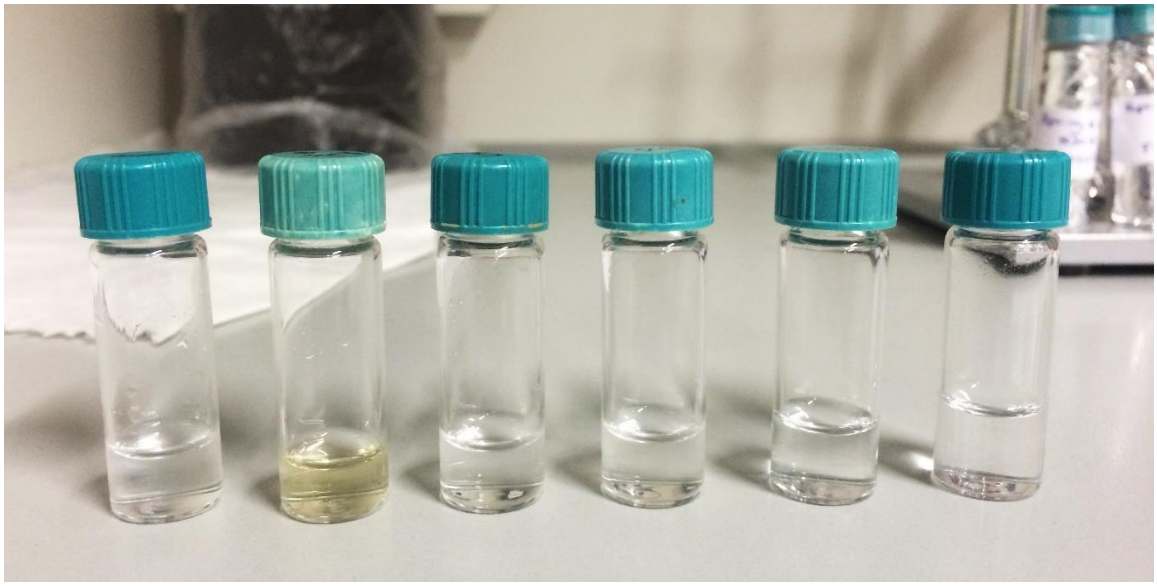


Figure 23 Vials of the samples after the filtration via a syringe filter. The vial number 2 corresponds to the filter with biologic material from bird activity.

6. Chromatographic instrumentation

The liquid chromatography equipment consisted of a JASCO quaternary low pressure gradient pump (model PU-2089 Plus), a Rheodyne injection valve (model 7725i) equipped with a 20 µL loop and one of the four used chromatographic columns: Acclaim® Mixed-Mode WAX-1 (Dionex, Sunnyvale, CA, USA; diameter 4.6 mm, length 150 mm, comprised of 5 µm high-purity, porous, spherical silica particles with 120 Å diameter pores bonded with alkyl amine functional groups); Acclaim® Mixed-Mode HILIC-1 (Dionex, Sunnyvale, CA, USA; diameter 4.6 mm, length 150 mm, comprised of 5 µm high-purity, porous, spherical silica particles with 120 Å diameter pores bonded with alkyl diol functional groups); Phenomenex® Luna HILIC (Phenomenex, CA, USA; diameter 4.6 µm, length 150 mm, comprised of 3.5 µm high-purity, porous, spherical silica particles with 200 Å diameter pores bonded to cross-linked diol groups); Phenomenex® Luna C18(2) (Phenomenex, CA, USA; diameter 4.6 µm, length 250 mm, comprised of 5 µm high-purity, porous, spherical silica particles with 100 Å diameter

pores bonded to octadecyl silane groups). The operating conditions varied and they are described in each study for the four columns in the section **III - Preliminary tests of standard amino acids solutions**. The temperature of the analytical column was maintained at 30 ºC. For the Acclaim® Mixed-Mode WAX-1, Acclaim® Mixed-Mode HILIC-1 and Phenomenex® Luna HILIC chromatographic columns, the oven in use was a JASCO column oven (model CO-2065 Plus). For the Phenomenex® Luna C18(2), a Phenomenex ThermaSphere column oven (model TS-130) was used.

The liquid chromatography equipment was connect to an evaporative light--scattering detector (SEDEX, model 80 LT-ELSD) operating at 60 ºC and 3.5 bar. The fluorescence spectra of each aerosol sample were recorded by means of a spectrophotometer JASCO FP-6500 using a 1 cm path-length quartz cuvette. Excitation wavelength ranges were set from 220 to 400 nm and emission wavelength ranges were set from 230 to 550 nm. The scanning intervals were set at 10 nm and 2 nm, respectively. The excitation and emission slit widths were set at 10 nm and the scan speed was 100 nm/min. The peaks due to water Raman scatter were eliminated from all spectra by subtracting the Milli-Q water blank spectra.

III

Preliminary tests of standard amino acids solutions

1. Aim of the preliminary tests

In order to study the influence of the mobile phase regarding the value of the pH, the concentration of the buffer and the concentration of the organic solvent in the amino acids (standard solutions) separation in each column, four different amino acids (a basic - lysine, an acidic – aspartic acid, a neutral – glycine, and an aromatic - tryptophan) were eluted in distinct chromatographic conditions. The standard solutions were prepared with individual *L*-amino acids.

2. Results of the preliminary tests on the Acclaim® Mixed-Mode WAX-1 column

The experiments conducted in this column aimed at assessing the effect of both the ionic strength and the amount of organic solvent on the retention behavior of the four selected amino acids. The individual amino acids were dissolved in ultra-pure water and in the mobile phase (ammonium acetate with acetic acid and MeOH) in the proportion of 70:30 (V/V). The conditions that were kept constant in the chromatographic separation of the amino acids and are shown in **Table 9**. The values of the liquid chromatography elution were normalized.

Table 9 Conditions of the preliminary tests for the Acclaim® Mixed-Mode WAX-1 column.

Conditions of the chromatographic separation			
pH:	4.00	Oven temperature:	30ºC
Buffer composition:	Ammonium acetate with acetic acid	Flow rate:	0.500 mL/min
Organic solvent:	MeOH		

2.1 Influence of the organic solvent concentration of the mobile phase in the elution of an acidic amino acid: aspartic acid

It was intended to study the effect of the variation of the organic solvent concentration at different buffer concentrations in the elution of an acidic amino acid, in the Acclaim® Mixed-Mode WAX-1 column. The results are shown in **Figure 24**.

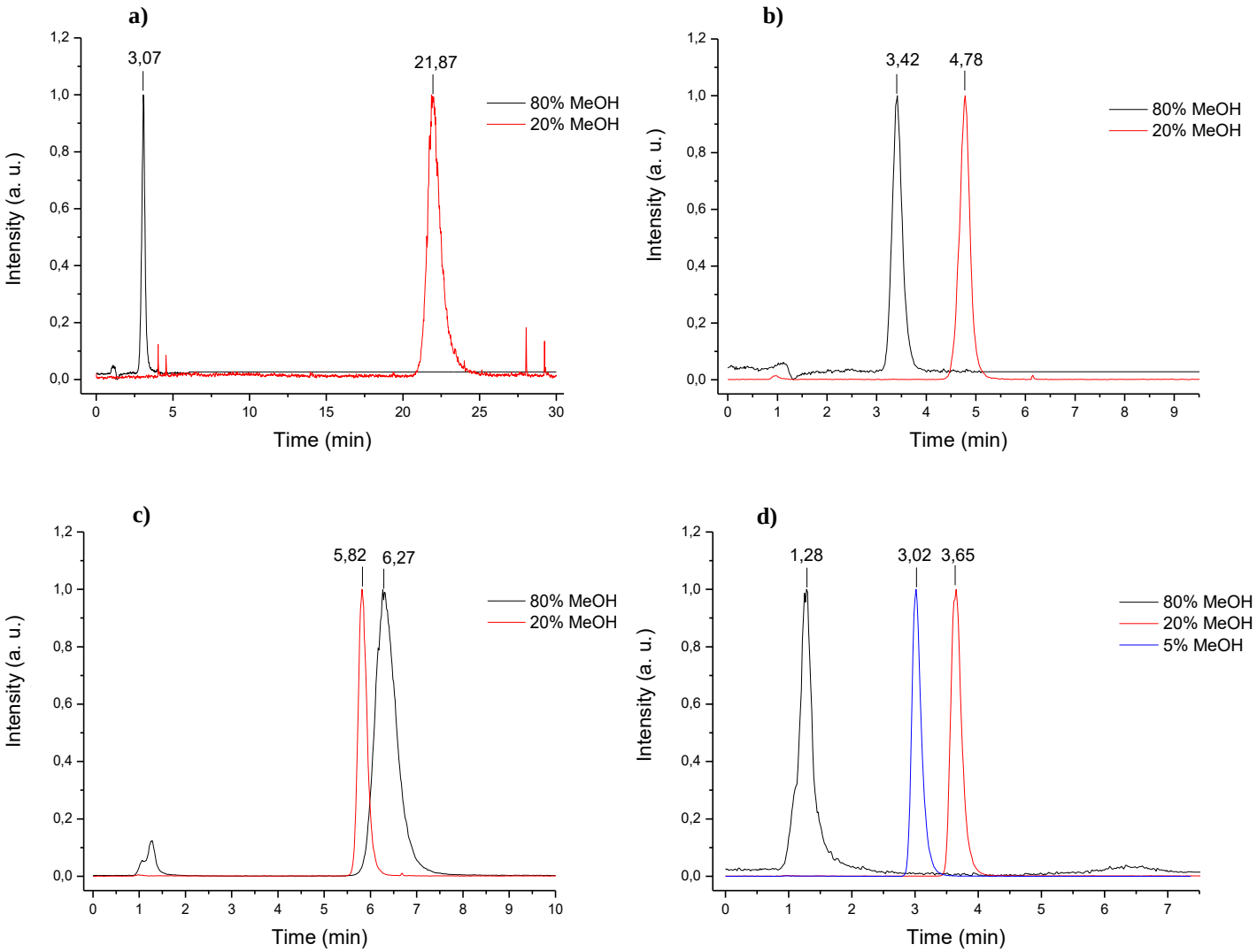


Figure 24 Chromatograms showing the effect of the variation of the organic solvent concentration at different buffer concentrations - **a)** 20mM; **b)** 50mM; **c)** 100mM; **d)** 200mM - in the elution of the aspartic acid.

In **Figure 24 - a)** it is possible to observe that the retention time increases with the decrease of the organic solvent content in the mobile phase. The increase of the organic solvent concentration to 80% (V/V) reduces the retention time of the hydrophilic aspartic

acid due to the alkyl chains in the stationary phase. The alkyl chains are hydrophobic, resulting in a low interaction between the stationary phase and the aspartic acid, a hydrophilic amino acid. Thus, this analyte tends to interact more with the mobile phase, which leads to a decrease of the retention time. The same situation occurs in **Figure 24 – b)**: the increase of organic solvent content in the mobile phase led to a decrease in the retention of the aspartic acid, which can be explained by the hydrophilicity of the different phases. With 80% (V/V) of organic solvent, the mobile phase is more hydrophobic than the stationary phase, resulting in a shorter elution of the aspartic acid. Still, the shift in the retention time was higher for a lower ionic strength (**Figure 24 – a)**), i.e., – for a buffer concentration of 20mM.

In **Figure 24 – c)**, there is an opposite trend in the retention time of aspartic acid in comparison to those of **Figure 24 – a)** and **Figure 24 – b)**. For a buffer concentration of 100mM, the increase in the amount of organic solvent in the mobile phase resulted in an increase of the retention time of the aspartic acid. In this situation, with a buffer concentration of 100mM, the organic solvent concentration may not be the only property influencing the retention of the amino acid – the ionic strength also has to be considered. In **Figure 24 – d)**, and for a buffer concentration of 200mM, the aspartic acid was eluted at the column void volume when the organic solvent content is at 80% (V/V). When using lower contents of organic solvent (5% (V/V) and 20% (V/V)) with a 200mM buffer concentration, it is possible to observe an increase of the retention of the aspartic acid when the organic solvent also increases. In **Figure 24 – c)** and **d)**, with a buffer concentration of 100mM and 200mM, respectively, it is possible that more than one retention mechanisms are functioning at the same time, which results in a competition among the anion-exchange, RP and HILIC modes, leading to an increase of the retention time when the organic solvent concentration also increases.

2.2 Influence of the organic solvent concentration of the mobile phase in the elution of a basic amino acid: lysine

The retention behavior of the basic amino acid lysine was also assessed for different mobile phase compositions. The variation of the organic solvent concentration

at different buffer concentrations in the elution of this basic amino acid in the Acclaim® Mixed-Mode WAX-1 column was under study. The results are shown in **Figure 25**.

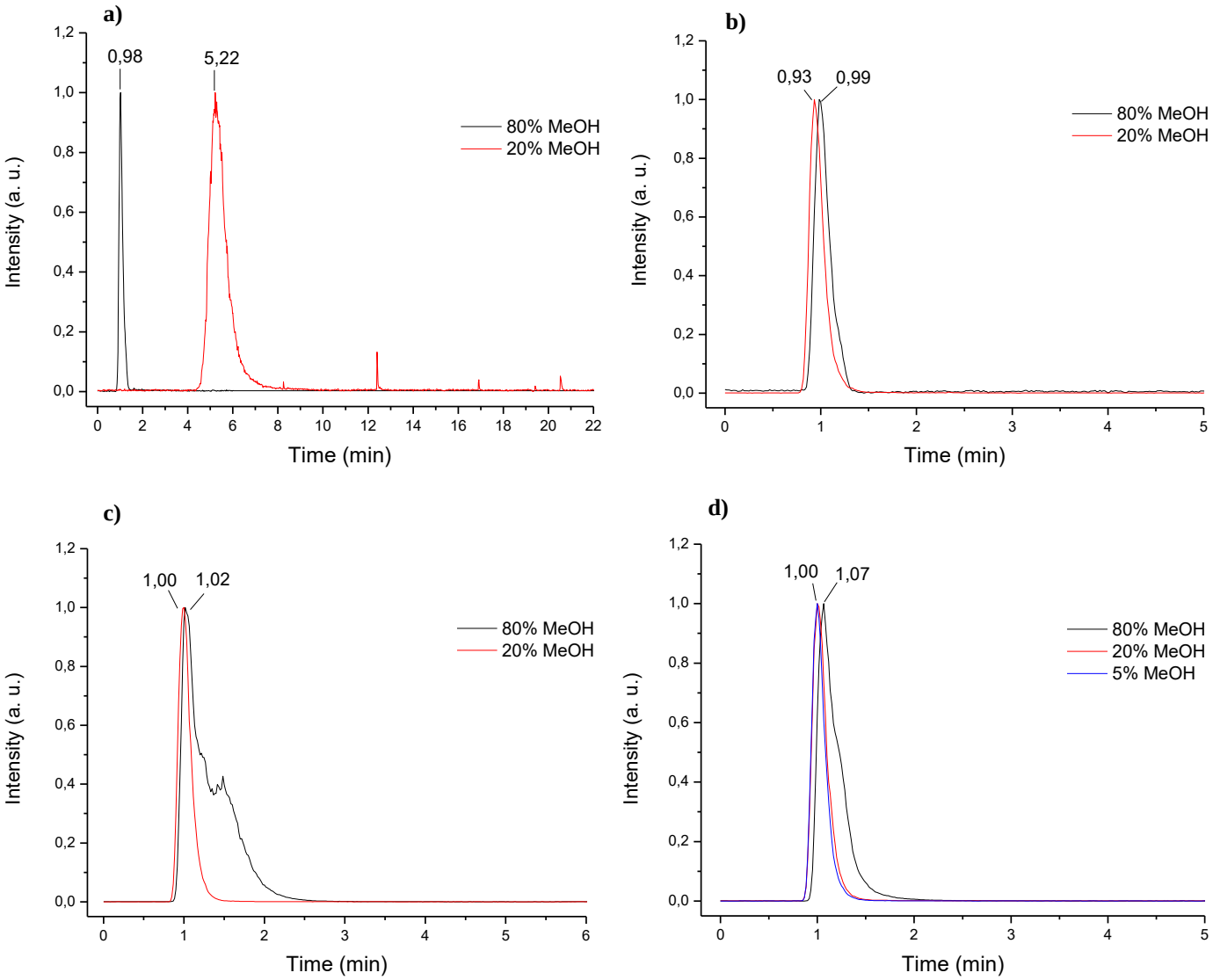


Figure 25 Chromatograms showing the effect of the variation of the organic solvent concentration at different buffer concentrations - **a)** 20mM; **b)** 50mM; **c)** 100mM; **d)** 200mM - in the elution of lysine.

The chromatograms in **Figure 25** shows that lysine elutes at the column void volume, except when using a mobile phase consisting of 20mM ammonium acetate and 20% (V/V) MeOH (**Figure 25 – a)**). Taking into account these results, it is impossible to conclude about the behavior and the influence of the buffer concentration in this column, when studying the lysine amino acid.

2.3 Influence of the organic solvent concentration of the mobile phase in the elution of a neutral amino acid: glycine

In **Figure 26**, the results for the study of the variation of the organic solvent concentration at different buffer concentrations in the elution of a neutral amino acid in the Acclaim® Mixed-Mode WAX-1 column are shown.

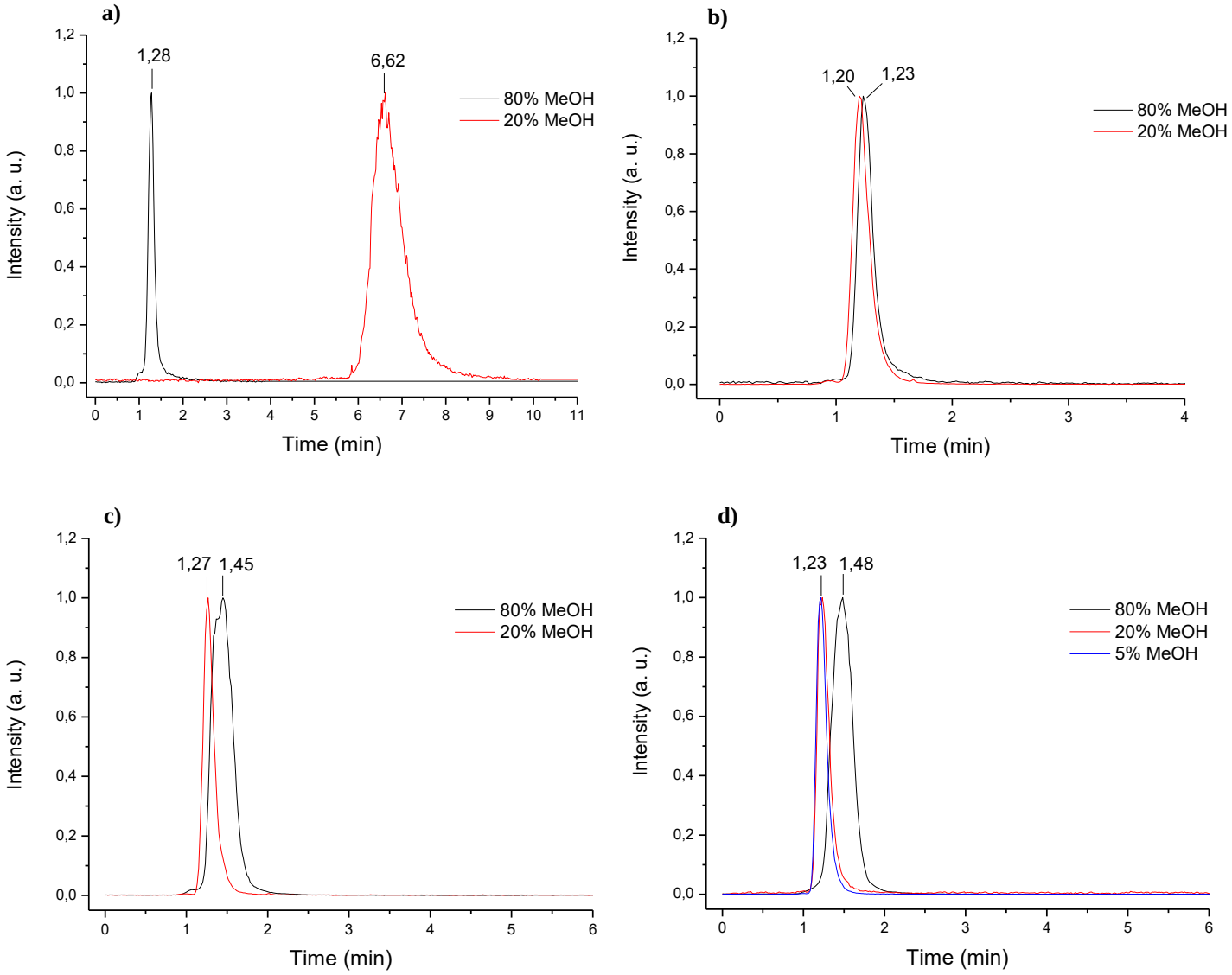


Figure 26 Chromatograms showing the effect of the variation of the organic solvent concentration at different buffer concentrations - **a)** 20mM; **b)** 50mM; **c)** 100mM; **d)** 200mM - in the elution of glycine.

In the first chromatogram, **Figure 26 - a)**, the retention time of the molecule increased with the decrease of the percentage of the organic solvent in the mobile phase. With 80% (V/V) of organic solvent concentration, the hydrophilic analyte, glycine, interacts more with the mobile phase due to the hydrophobic alkyl chains in the stationary phase of the column, leading to a shorter elution. In the second chromatogram, **Figure 26 – b)**, the retention times for the different conditions are very similar, which does not allow any conclusion regarding the influence of the organic solvent concentration in the mobile phase and the retention of glycine, for a concentration buffer of 50mM.

In the third case, **Figure 26 – c)**, there was an inversion regarding the relation of the amount of organic solvent with the retention of the amino acid when compared to the chromatograms in **Figure 26 – a)** and **b)**. In **Figure 26 – c)**, the increase of the volume of the organic solvent in the mobile phase led to an increase of the retention time of glycine – in the elution with a buffer concentration of 100mM, the organic solvent concentration in the mobile phase is not the only condition that affects the chromatographic elution but also the ionic strength of the mobile phase. The same situation is seen in the last chromatogram, **Figure 26 – d)**, in which, for a buffer concentration of 200mM, the analyte was slightly more eluted in 80% of MeOH than in the lower percentages of organic solvent. In the elutions with 5% (V/V) and 20% (V/V) of organic solvent, the retention times were very similar and did not show any relevant difference between the peaks. In **Figure 26 – c)** and **d)**, with a buffer concentration of 100mM and 200mM, respectively, more than one retention mechanism may have been functioning simultaneously. Both ionic strength and organic solvent concentration were influencing the elution of glycine, leading to a competition among the anion-exchange, HILIC and RP modes, which resulted in an increase of the retention time when the organic solvent concentration also increases.

2.4 Influence of the organic solvent concentration of the mobile phase in the elution of an aromatic amino acid: tryptophan

It was intended to study the effect of the variation of the organic solvent concentration at different buffer concentrations in the elution of an aromatic amino acid, in the Acclaim® Mixed-Mode WAX-1 column. The results are shown in **Figure 27**.

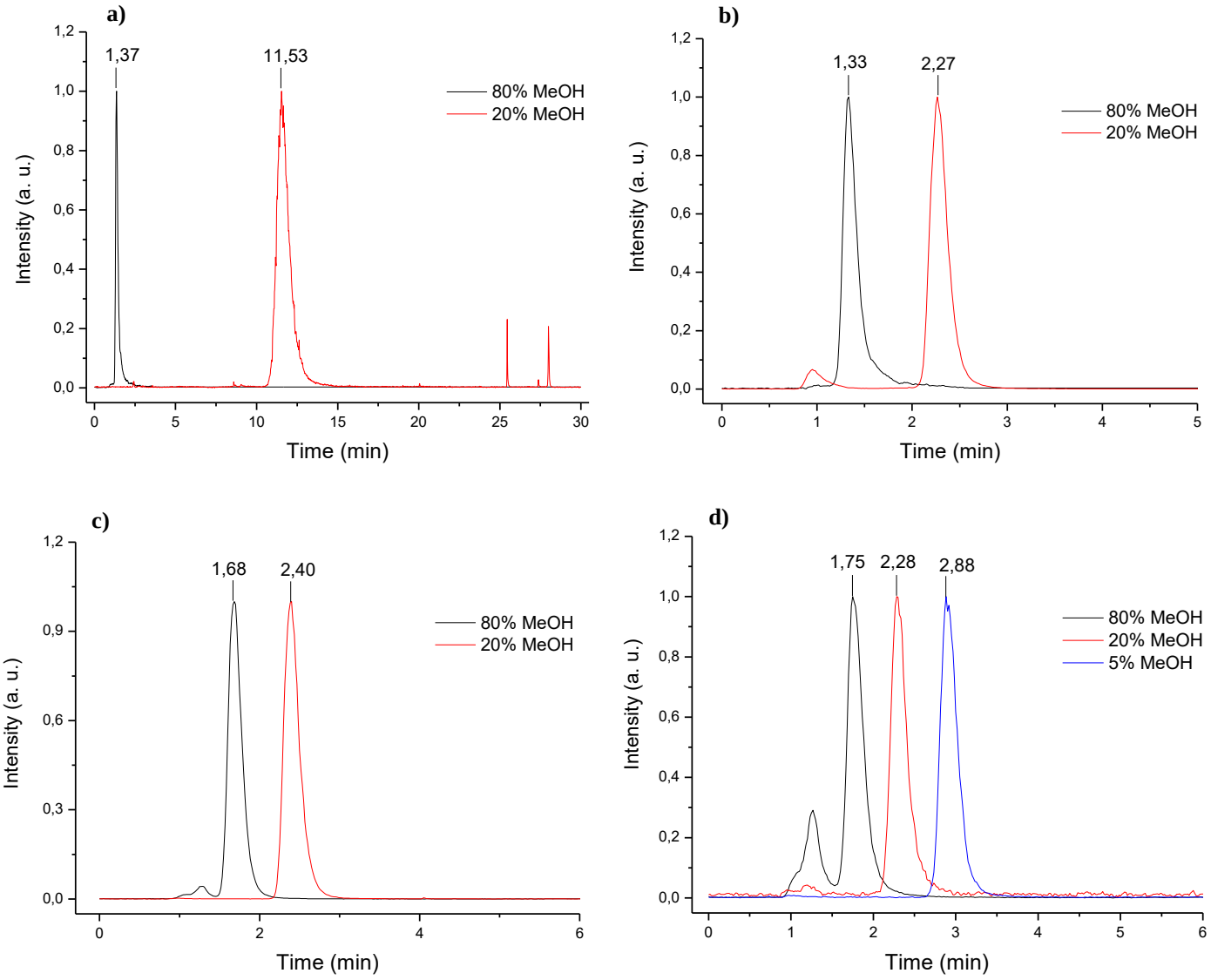


Figure 27 Chromatograms showing the effect of the variation of the organic solvent concentration at different buffer concentrations - **a)** 20mM; **b)** 50mM; **c)** 100mM; **d)** 200mM - in the elution of tryptophan.

In **Figure 27 - a)**, it is possible to verify that tryptophan elutes longer when the mobile phase has a lower content of organic solvent. The same trend occurs in the other

three chromatograms, **Figure 27 – b)**, **c)** and **d)**, confirming a constant relation between the amount of organic solvent and the elution behavior of tryptophan, independently of the buffer concentration. Increasing the organic solvent concentration in the mobile phase leads to a shorter interaction between the hydrophilic analyte, tryptophan, and the alkyl chains in the stationary phase of the column, resulting in a lower retention time. Considering the four chromatograms, **Figure 27 – a)**, **b), c)** and **d)**, it is possible to observe the ionic strength does not influence the elution of tryptophan in the Acclaim® Mixed-Mode WAX-1 column.

Although the sample that was injected only had one compound, it is possible to notice more than one peak in the following chromatograms: 20% of MeOH when the buffer concentration is 50mM (**Figure 27 – b)**), 80% of MeOH when the buffer concentration is 100mM (**Figure 27 – c)**), 20% and 80% of MeOH when the buffer concentration is 200mM (**Figure 27 – d)**); which can indicate the vials where the samples were prepared were not properly cleaned.

2.5 Influence of the ionic strength of the mobile phase in the elution of an acidic amino acid: aspartic acid

It was intended to study the effect of the variation of the buffer concentration at different organic solvent concentrations in the elution of an acidic amino acid, in the Acclaim® Mixed-Mode WAX-1 column. The results are shown in **Figure 28**.

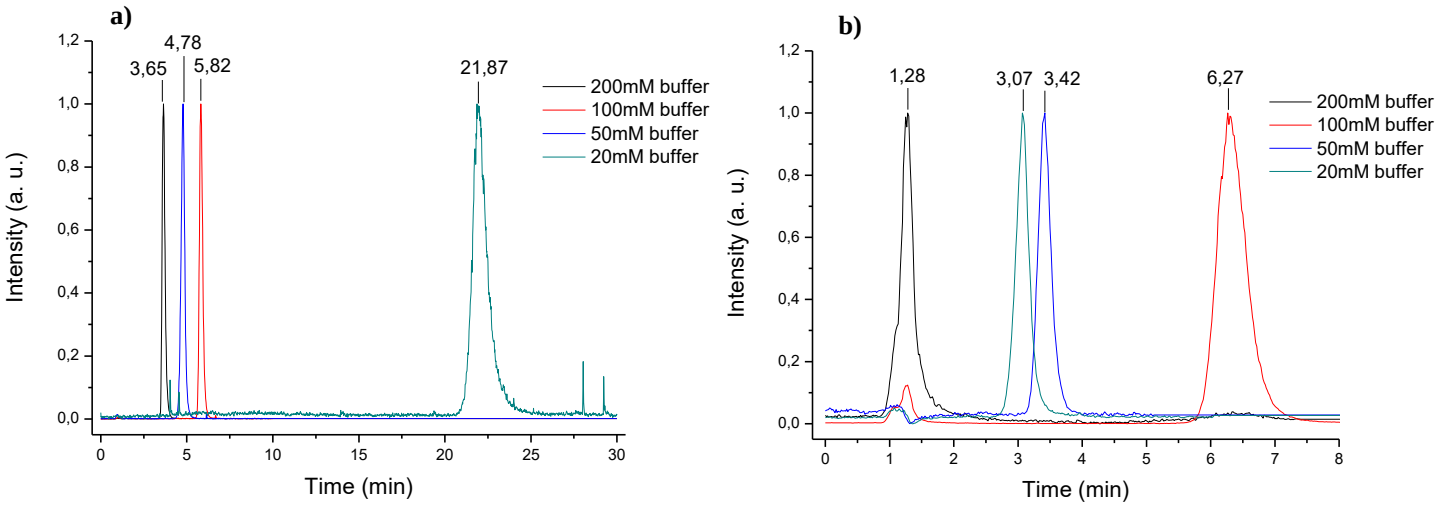


Figure 28 Chromatograms showing the effect of the variation of the ionic strength at different organic solvent concentrations - **a)** 20%; **b)** 80% - in the elution of the aspartic acid.

In **Figure 28 – a)**, although the retention time of the aspartic acid is higher for the lowest buffer concentration and lower for the highest buffer concentration, the retention time of the aspartic acid at a buffer concentration of 50mM is lower than that obtained at a buffer concentration of 100mM, which does not allow any conclusion regarding the effect of the ionic strength in the elution of the aspartic acid. Considering the Acclaim® Mixed-Mode WAX-1 has three different retention mechanisms, at an elution with 20% (V/V) of organic solvent, **Figure 28 – a)**, all of them, anion-exchange, RP and HILIC, can be competing among each other when the buffer concentrations of the elutions are 50mM and 100mM, leading to an elution that is affected by both the ionic strength and the organic solvent concentration. The increase of the ionic strength indicates that there are more ions in the mobile phase, both cations and anions. The anions in the mobile phase will interact more with the stationary phase since there is an exchange of the negatively charged molecules: the anions of the mobile phase will occupy the position of the anions of the stationary phase surface. The opposite occurs for the positively charged particles: the interaction of the ionized acid with the stationary phase decreases with the increase of the amount of charged particles in the mobile phase, since there is no cationic exchange, which explains the long elution of the aspartic acid in the buffer concentration of 20mM, **Figure 28 – a)**.

In **Figure 28 – b)**, the lowest retention time is obtained for the highest buffer concentration, 200mM, which did not happen in the cases of a buffer concentration of 20mM, 50mM and 100mM. Except for the case of 200mM buffer concentration, the increase of the ionic strength from 20mM to 100mM led to an increase of the retention of the aspartic acid. Considering the fact that at 80% (V/V) of organic solvent in the mobile phase the column operates under the HILIC mode, this mode may become the driving force for the separation of aspartic acid for buffer concentrations lower than 200mM. Therefore, for the elutions with buffer concentrations of 20mM, 50mM and 100mM, in **Figure 28 – b)**, the elution profile of the aspartic acid is likely of being influenced by the HILIC mode, thus yielding another conclusion: in the HILIC mode, the increase of the organic solvent content increased the retention of a polar molecule (the aspartic acid is a polar amino acid).

2.6 Influence of the ionic strength of the mobile phase in the elution of a basic amino acid: lysine

The retention behavior of lysine was assessed for different mobile phase compositions: the variation of the ionic strength at different organic solvent concentrations in the elution of a basic amino acid in the Acclaim® Mixed-Mode WAX-1 column was under study. The results are shown in **Figure 29**.

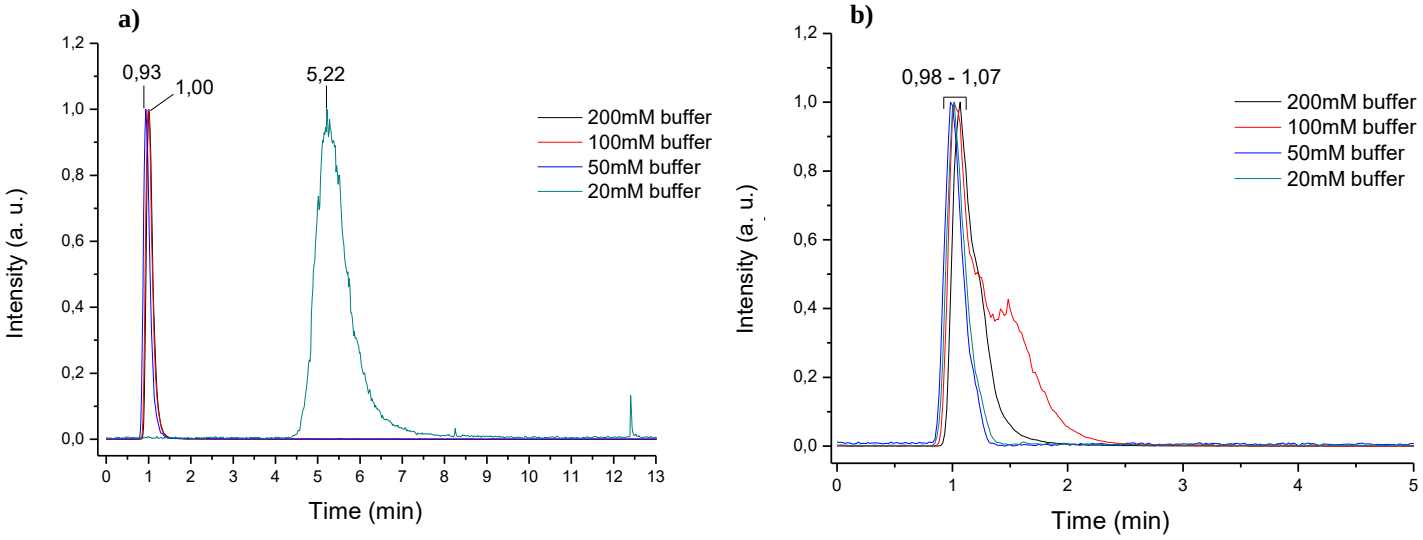


Figure 29 Chromatograms showing the effect of the variation of the ionic strength at different organic solvent concentrations - **a)** 20%; **b)** 80% - in the elution of lysine.

Figure 29 shows that in seven out of the eight applied elution conditions, lysine is eluting at the column void volume. Only in the first chromatogram, in **Figure 29 – a)**, when using a buffer concentration of 20mM, it was possible to get a sufficient retentivity of lysine, which does not allow any conclusion regarding the influence of the ionic strength in the elution of lysine.

2.7 Influence of the ionic strength of the mobile phase in the elution of a neutral amino acid: glycine

For the study of the effect of the variation of the ionic strength in a neutral amino acid in the Acclaim® Mixed-Mode WAX-1 column, glycine was eluted in distinct buffer concentrations at different organic solvent concentrations. The results are shown in **Figure 30**.

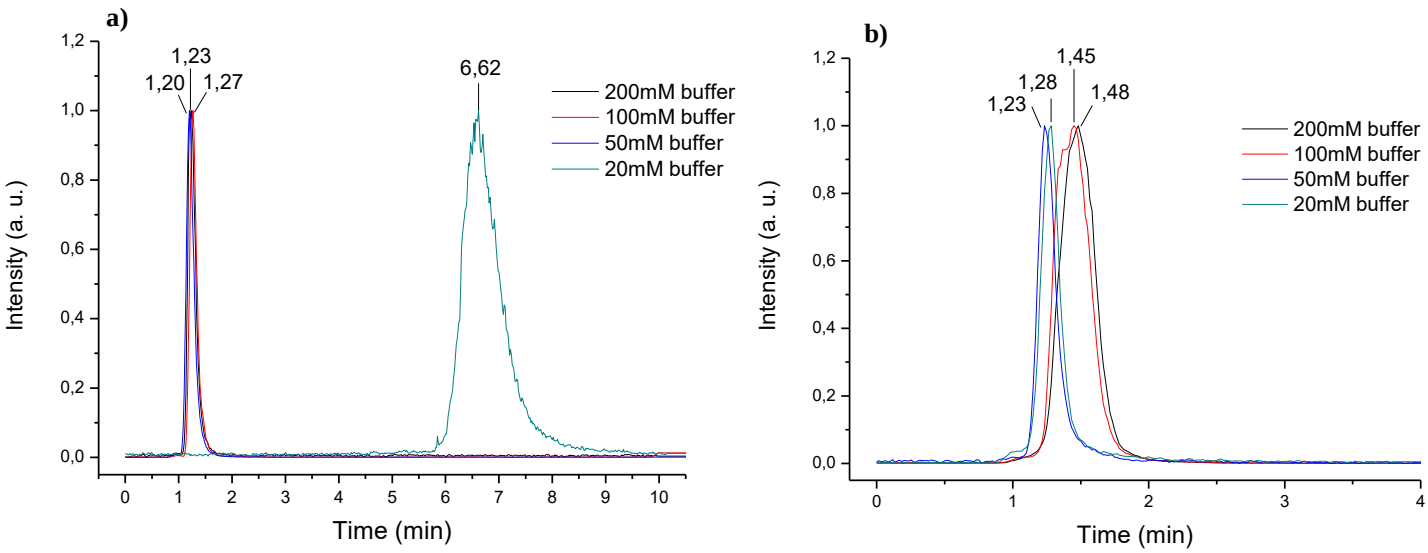


Figure 30 Chromatograms showing the effect of the variation of the ionic strength at different organic solvent concentrations - **a)** 20%; **b)** 80% - in the elution of glycine.

In **Figure 30 – a)**, it is possible to observe that, for buffer concentrations of 200mM, 100mM, and 50 mM, the glycine is eluting at approximately the same retention time. This indicates that, for buffer concentrations equal to or less than 50mM, the increase of the ionic strength does not have any influence in the elution of glycine when the organic solvent concentration is 20% (V/V). In this conditions, with 20% (V/V) of organic solvent, the three retention mechanisms, anion-exchange, RP and HILIC, are functioning, which indicates that both the ionic strength and the organic solvent concentration are influencing the elution of glycine. In **Figure 30 – a)**, only at a buffer concentration of 20mM it is possible to verify an interaction between the glycine and the stationary phase, since the analyte is eluting at a retention time away from the column void volume.

In **Figure 30 – b)**, and regardless of the employed mobile phase composition, it is possible to verify that glycine is eluting at retention time values very close to each other, indicating that the variation of the ionic strength does not influence the elution of glycine, when the organic solvent concentration is 80% (V/V) in the Acclaim® Mixed--Mode WAX-1 column.

2.8 Influence of the ionic strength of the mobile phase in the elution of an aromatic amino acid: tryptophan

It was intended to investigate the effect of the variation of the buffer concentration at different organic solvent concentrations in the elution of tryptophan, an aromatic amino acid, in the Acclaim® Mixed-Mode WAX-1 column. The results are shown in **Figure 31**.

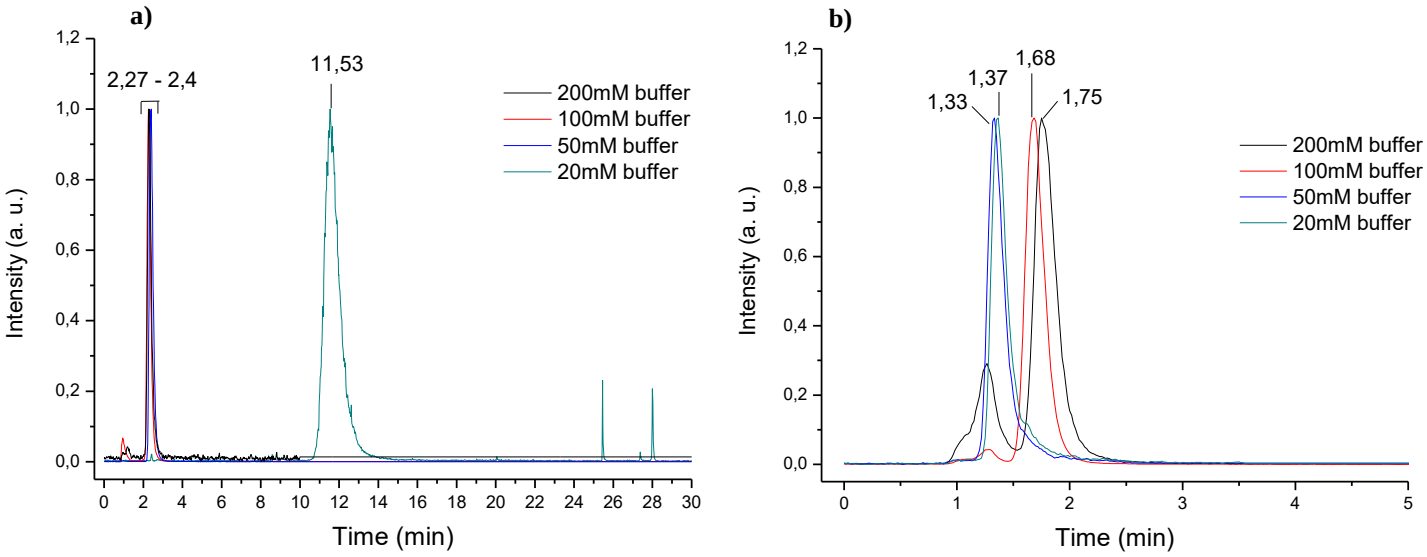


Figure 31 Chromatograms showing the effect of the variation of the ionic strength at different organic solvent concentrations - **a)** 20%; **b)** 80% - in the elution of tryptophan.

In **Figure 31 – a)**, it is possible to verify that, for buffer concentrations of 200mM, 100mM and 50mM, the tryptophan is eluted at approximately the same retention time,

indicating that, for buffer concentrations equal or higher than 50mM, increasing the ionic strength does not have any effect in the elution of tryptophan, when the organic solvent concentration is 20% (V/V). When the mobile phase has 20% (V/V) of organic solvent, all the three mechanisms, HILIC, RP and anion-exchange, are functioning, revealing that there is more than one condition that is influencing the elution: both ionic strength and organic solvent concentration have an effect on the elution of tryptophan.

In **Figure 31 – b)**, it is possible to notice the proximity between the peaks of the 20mM and the 50mM elutions, and the peaks of the 100mM and 200mM elutions. This reveals that, for an organic solvent concentration of 80% (V/V), tryptophan is slightly less eluted when the buffer concentration is equal to or less than 50mM, indicating an influence of the buffer concentration in the elution of this analyte. Although there is a slight difference between the 20mM and 50mM peaks and the 100mM and 20mmM peaks, that difference is not significant enough to get any information from the results, not allowing the achievement of any conclusions about the effect of the ionic strength in the elution of tryptophan in the Acclaim® Mixed-Mode WAX-1 column.

3. Results of the preliminary tests on the Acclaim® Mixed-Mode HILIC-1

3.1 Influence of the organic solvent concentration of the mobile phase in the elution behavior of four different amino acids

For assessing the effect of the organic solvent concentration in the elution behavior of the aspartic acid, lysine, glycine and tryptophan, the individual amino acids were dissolved in ultra-pure water and in the mobile phase (ammonium acetate with acetic acid and ACN), in the proportion of 70:30 (V/V), and eluted in different organic solvent concentrations. The conditions that were kept constant in the chromatographic separation of the amino acids are shown in **Table 10**. The values of the liquid chromatography elution in **Figure 32** were normalized.

Table 10 Conditions of the preliminary tests for the Acclaim® Mixed-Mode HILIC-1 column in order to study the influence of the organic solvent concentration in the retention of the aspartic acid, lysine, glycine and tryptophan.

Conditions of the chromatographic separation			
pH:	5.45	Oven temperature:	30ºC
Buffer composition:	Ammonium acetate with acetic acid, 50 mM	Flow rate:	0.500 mL/min
Organic solvent:	ACN		

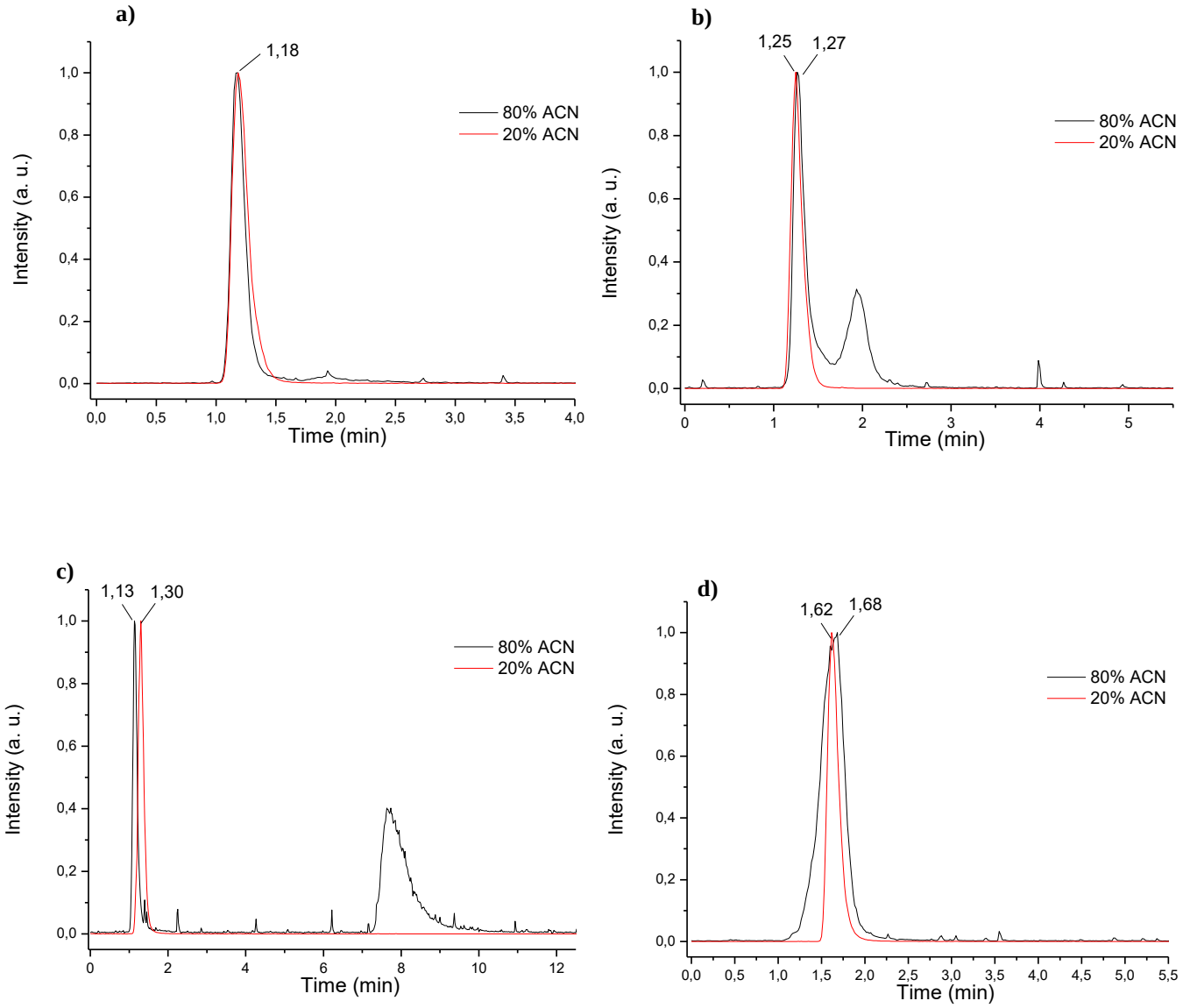


Figure 32 Chromatograms showing the effect of the variation of the organic solvent concentration at a buffer concentration of 50mM in the retention behaviour of: **a)** aspartic acid; **b)** lysine; **c)** glycine; **d)** tryptophan.

Although there is a significant difference in the composition of the mobile phase regarding the organic solvent content, there is no relevant variation on the retention time of the analytes when the percentage of ACN changes, as seen in **Figure 32**. When using an amount of organic solvent higher than 90% (V/V), the Acclaim® Mixed-Mode HILIC-1 column is operating under the HILIC mode. On the other hand, for organic solvent content lower than 75% (V/V), the stationary phase is operating under RP conditions. Thus, a mobile phase with 80% (V/V) of ACN is not enough to guarantee that the column is operating in the HILIC mode, which may hinder the usual influence of the organic solvent in this type of stationary phases: in the HILIC mode, the increase of the organic solvent concentration in the mobile phase should lead to an increase of the retention of the polar molecules (such as aspartic acid: **Figure 32 – a)**, and lysine: **Figure 32 – b)**). The opposite trend should occur for the nonpolar molecules (such as glycine: **Figure 32 – c)**, and tryptophan: **Figure 32 – d)**): the increase of the organic solvent concentration leads to a decrease of the polarity of the mobile phase, resulting in an increase of the interactions of the nonpolar analytes with the mobile phase. Since there are no significant changes in the retention time of the studied amino acids with the variation of the organic solvent content in the mobile phase, it is not possible to achieve any conclusion about the effect of the amount of organic solvent in the elution of the amino acids in the Acclaim® Mixed-Mode HILIC-1 column. Nevertheless, when the mobile phase has more content of ACN than of the aqueous buffer, the S/N ratio is higher than in the case of a small volume of the organic solvent. In the chromatograms **b)** and **c)**, there is more than one peak, revealing that the analytes, lysine and glycine, respectively, may have suffered the influence of both adsorption and repulsion phenomena, competing against each other.

3.2 Influence of the preparation of the amino acids solutions in the elution of four different amino acids

In order to assess the influence of the amino acids solutions preparation in the retention behavior of the amino acids, the amino acids were dissolved in a different solution from the mobile phase, not containing any organic solvent. For this study, the

individual amino acids were dissolved in ultra-pure water and buffer solution (ammonium acetate with acetic acid), in the proportion of 70:30 (V/V). The conditions that were kept constant for the following analysis are shown in the **Table 11**. The values of the liquid chromatography elution were normalized and are shown in **Figure 33**.

Table 11 Conditions of the preliminary tests for the Acclaim® Mixed-Mode HILIC-1 column in order to study the influence of the amino acids solution preparation in the retention of the aspartic acid, lysine, glycine and tryptophan.

Conditions of the chromatographic separation

pH:	5.45	Oven temperature:	30ºC
Buffer composition:	Ammonium acetate with acetic acid, 50 mM	Flow rate:	0.500 mL/min
Organic solvent:	ACN		

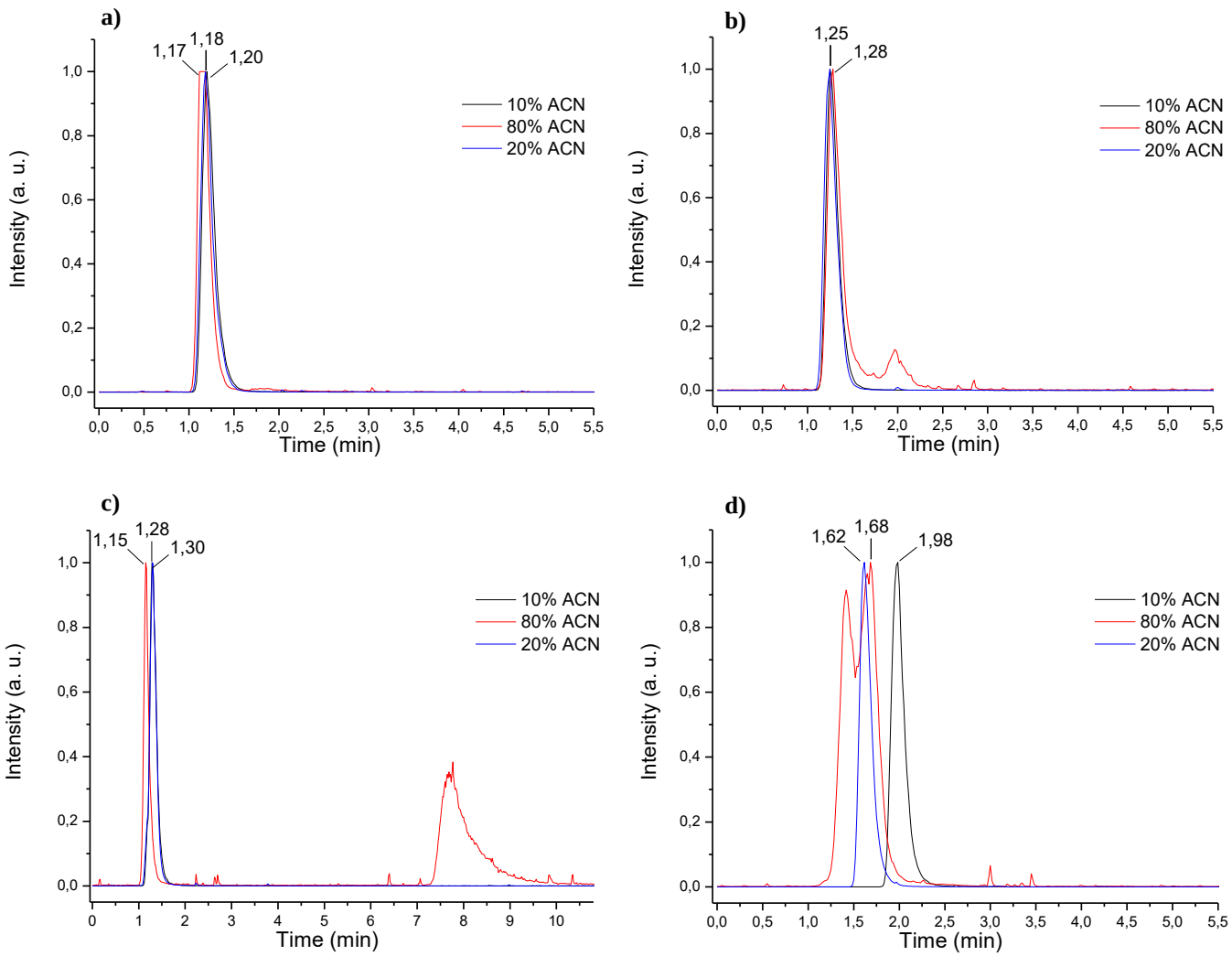


Figure 33 Chromatograms of the four tested amino acids eluted under different organic solvent content conditions: **a)** aspartic acid; **b)** lysine; **c)** glycine; **d)** tryptophan.

Regardless of the amount of ACN in the mobile phase, is it possible to observe, in **Figure 33**, that there is no relevant change in the elution behavior of the aspartic acid, glycine, lysine and tryptophan. According to the conditions of the chromatographic elution, 10% (V/V), 20% (V/V) and 80% (V/V) of organic solvent concentration, the Acclaim® Mixed-Mode HILIC-1 column is operating in the RP mode. In the chromatograms of lysine (**Figure 33 – b)**) and glycine (**Figure 33 – c)**), there is more than peak, revealing the possibility of a competition between the two retention mechanisms, HILIC and RP, although the organic solvent concentration is always lower than 90% (V/V) – minimum value to ensure the HILIC mode. Taking in consideration that there are no significant differences in the retention time of the amino acids in the chromatograms of the **Figure 33**, it is not possible to take any information regarding the influence of the organic solvent concentration in the elution of the aspartic acid, glycine, lysine and tryptophan, when the analytes are dissolved in a solution without the organic solvent of the mobile phase. In **Figures 33 – b)**, **c)** and **d)**, it is possible to verify that the S/N ration is higher for an organic solvent concentration of 80% (V/V) than 10% (V/V) or 20% (V/V).

3.3 Influence of the organic solvent concentration of a mobile phase without buffer in the elution behavior of four different amino acids

Aiming the assessment of the influence of the organic solvent concentration in the retention of the amino acids, in a mobile phase without buffer, the individual amino acids were dissolved only in acidified water (pH=2.98, water acidified with formic acid - 0.025% (V/V)). The solution in which the amino acids were dissolved was different from the mobile phase since it did not contain any organic solvent. The conditions employed in these experiments are shown in **Table 12**. The values of the liquid chromatography elution were normalized and are shown in **Figure 34**.

Table 12 Conditions of the preliminary tests for the Acclaim® Mixed-Mode HILIC-1 column to study the effect of the organic solvent content in the elution of the aspartic acid, lysine, glycine and tryptophan.

Conditions of the chromatographic separation

pH:	2.98	Oven temperature:	30ºC
Aqueous eluent:	Water acidified with formic acid	Flow rate:	0.500 mL/min
Organic solvent:	ACN		

For this study, the same amino acid was eluted under different mobile phase compositions, by changing the amount of organic solvent. The aqueous part of the mobile phase was only acidified water in an attempt to reproduce the mobile phase conditions applied by Noga *et al.* (2013), who used a gradient elution to separate all the analytes in a mixture with six amino acids (tryptophan, leucine, isoleucine, proline, threonine and glycine) and vitamin B6. The authors used a mobile phase with ACN (eluent A) and water acidified with formic acid (pH 3.00, eluent B) in the following gradient program - A:B (80:20) for 5 min to A:B (50:50) in 25min and held for 5min, A:B (80:20) in 5min.

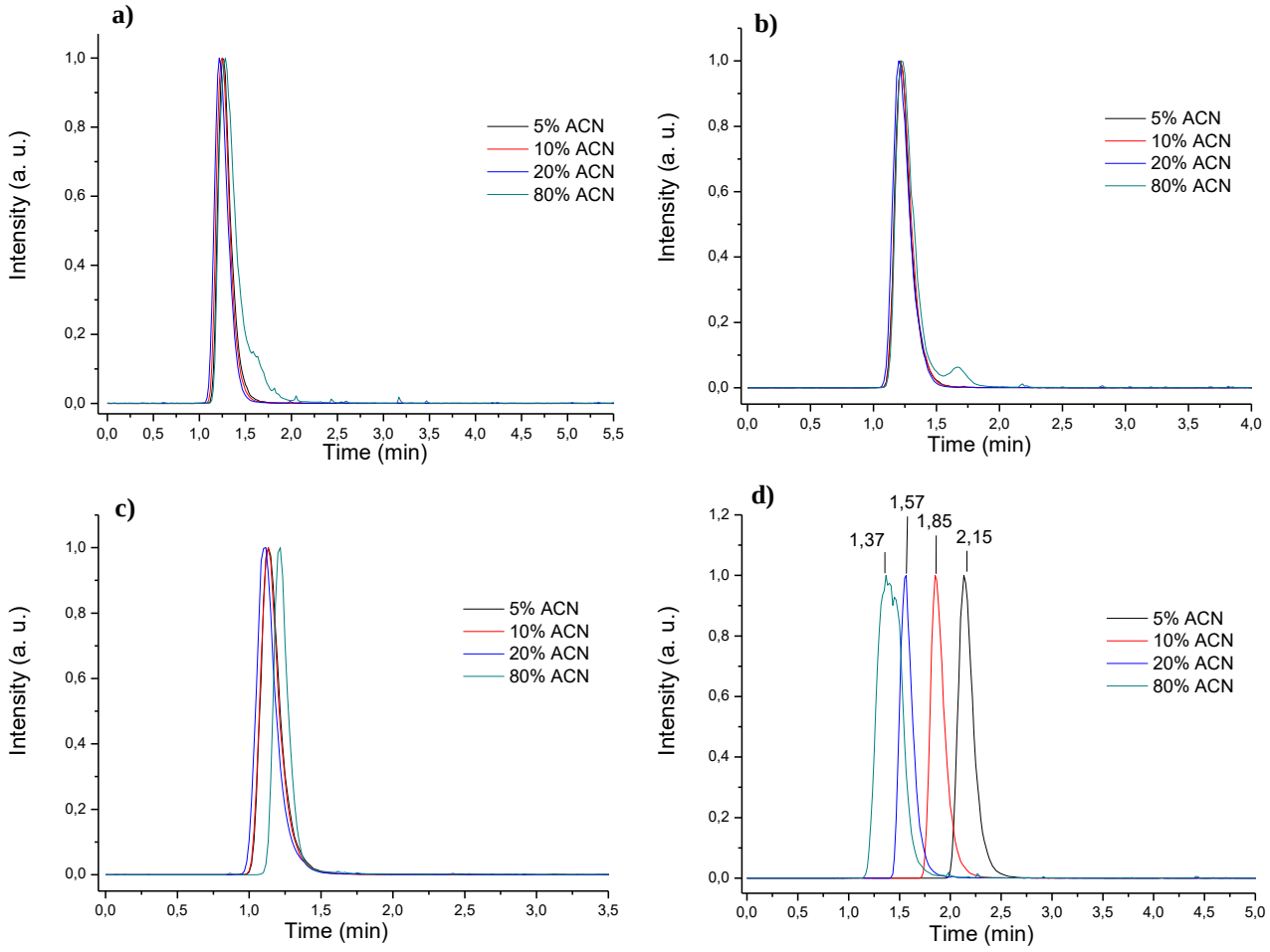


Figure 34 Chromatograms of the four amino acids eluted under different mobile phase conditions, in terms of the amount of ACN: **a)** aspartic acid; **b)** lysine; **c)** glycine; **d)** tryptophan.

Figure 34 shows that there is no significant difference in the elution behavior of the selected amino acids despite of the substantial variation of the organic solvent content in the mobile phase. Although there is a slight difference in the retention time of tryptophan in **Figure 34 – d)**, the variation is not relevant enough to ensure it was the result of the influence of the organic solvent concentration variation. Thus, it is not possible to establish any conclusion about the effect of the samples preparation in the elution for the chromatographic conditions in **Table 12**. Moreover, these conditions (mobile phase: ACN and water acidified with formic acid, pH 2.98) led to similar results there was a buffer in the mobile phase and the pH was 5.45, as shown in **Figure 33**, which may indicate that, when trying to separate amino acids in the Acclaim® Mixed-Mode HILIC-1 column, the presence of a buffer and the pH value may not have any influence in the elution of the aspartic acid, lysine, glycine and tryptophan.

In the chromatogram of **Figure 34 – b)**, the case of lysine, the signal shows two joined peaks for the elution with 80% of organic solvent, which may reveal that the vial was not properly cleaned.

3.4 Reproduction of the work of Noga *et al.* (2013) to study the influence of the organic solvent concentration in the elution of different amino acids in a mobile phase without buffer

For this study, six different amino acids (glycine, proline, isoleucine, leucine, threonine and tryptophan) were eluted in 80% (V/V) of ACN in the mobile phase. The goal was to reproduce the work of Noga *et al.* (2013), since the authors were capable of separating six distinct amino acids (glycine, proline, isoleucine, leucine, threonine and tryptophan) in a mixture with seven compounds (the six amino acids and vitamin B6). The elution was held at an acidic pH of 3.0, which means that all amino acids were positively charged during the chromatographic separation. First, the authors did an isocratic elution of a mixture with seven different compounds with a mobile phase of 80% of ACN and 20% of acidified water (pH = 3.0, adjusted with formic acid).

In order to reproduce the work of Noga *et al.* (2013), the individual amino acids were dissolved in acidified water (pH=2.98, water acidified with formic acid - 0.025%

(V/V)) and ACN in the proportion of 70:30 (V/V). The amino acids were eluted at 80% (V/V), **Figure 35**, and 20% (V/V), **Figure 36**, of organic solvent. The conditions that were kept constant are shown in **Table 13**. In these cases, the values were not normalized, in order to observe the difference in the intensities for the different samples.

Table 13 Conditions of the preliminary tests for the Acclaim® Mixed-Mode HILIC-1 column in order to study the effect of the organic solvent concentration in the retention behavior of glycine, leucine, isoleucine, proline, threonine and tryptophan.

Conditions of the chromatographic separation			
pH:	2.98	Oven temperature:	30ºC
Aqueous eluent:	Water acidified with formic acid	Flow rate:	1.000 mL/min
Organic solvent	ACN		

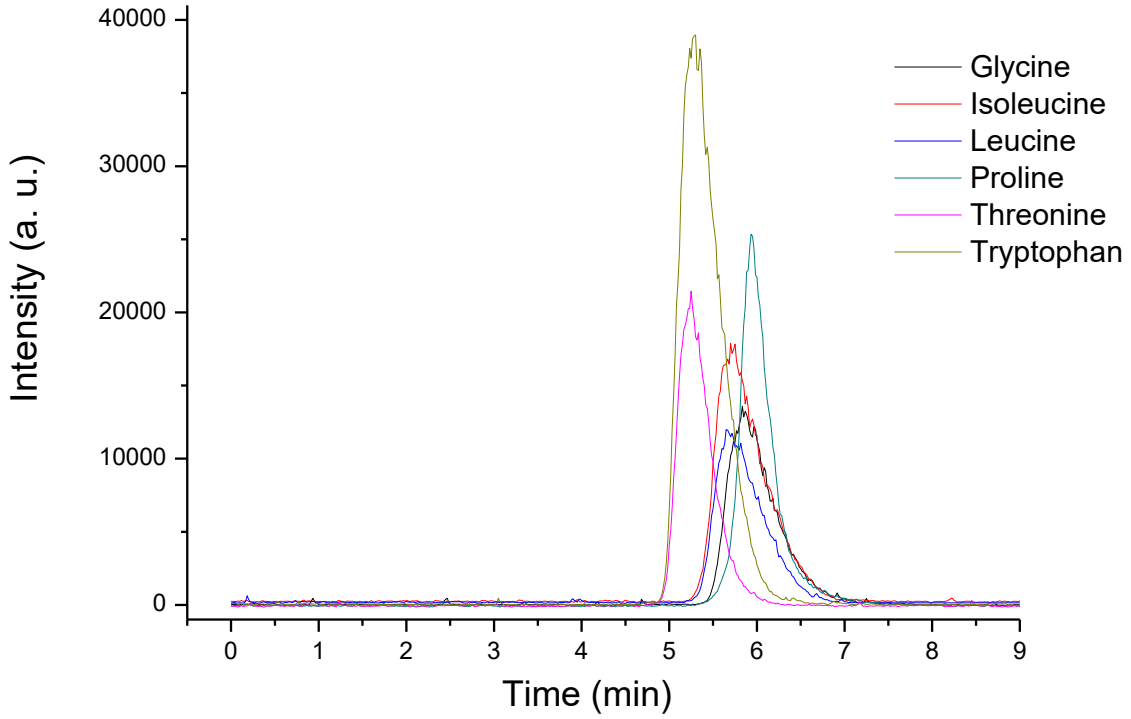


Figure 35 Results obtained with the isocratic elution of glycine, isoleucine, leucine, proline, threonine and tryptophan, using 80% (V/V) of organic solvent.

As shown in **Figure 35**, it was not possible to obtain an efficient separation of the six amino acids studied, in contrary to what has been reported by Noga *et al.* (2013). The analytes eluted between 5 and 7 minutes, contrary to the results obtained by Noga *et al.* (2013) – between 1 and 28 minutes. From the six studied amino acids, five were nonpolar

and one, threonine, was polar. Considering the fact that the amino acids were positively charged, there is an ionic interaction between the analyte and the stationary phase, which can explain the proximity of the retention times. Since all the molecules being studied are in the same ionic form, the interactions with the stationary phase are similar, not allowing an efficient separation.

Although both columns had HILIC functionalities and a diameter of 4.6mm, and considering that the samples had the same treatment and the mobile phase was similar to the one used by Noga *et al.* (2013), the divergence in the results may be a result of the difference in the stationary phase: it was used an Acclaim® Mixed-Mode HILIC-1 (diol groups) column while Noga *et al.* (2013) used a TSKgel NH2-100 (alkylamine groups).

Noga *et al.* (2013) also employed a gradient elution, varying the ACN and the acidified water concentrations in three values: 20% (V/V), 50% (V/V) and 80% (V/V). In order to understand the influence of the organic solvent content at a pH 2.98 in the elution of distinct amino acids, a chromatographic elution with 20% (V/V) of ACN was performed. With the intention to study the elution of acidic, basic and aromatic amino acids, additional amino acids were also studied.

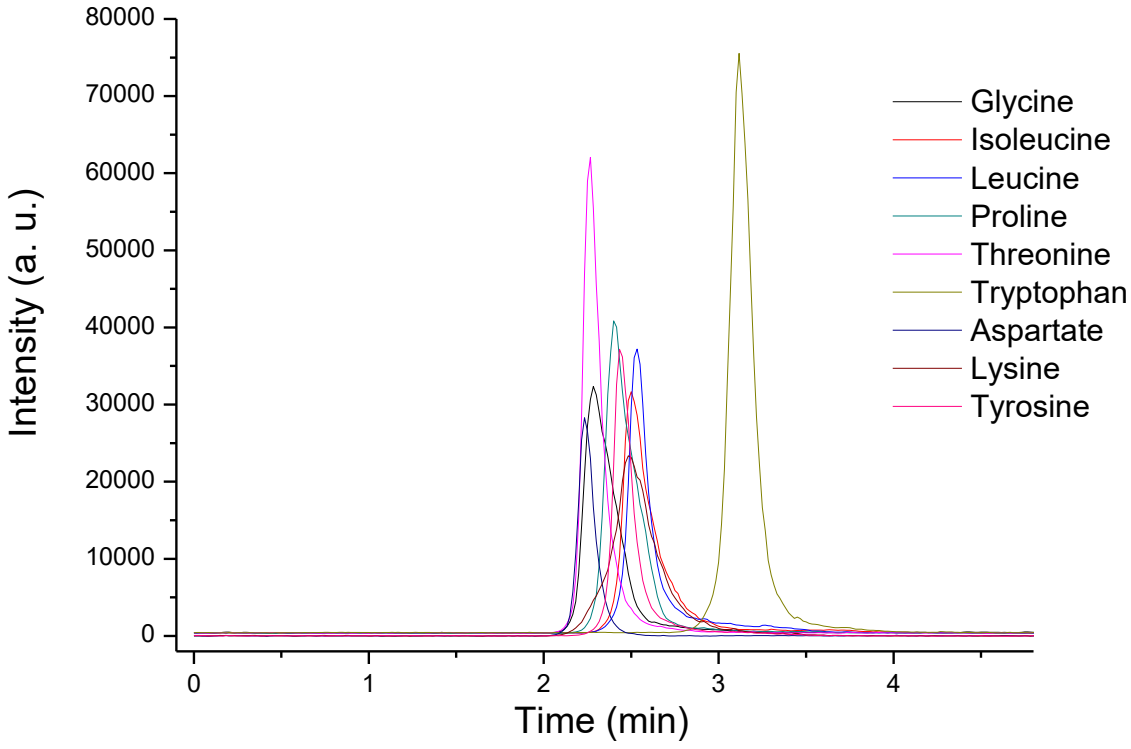


Figure 36 Results obtained for the isocratic elution of glycine, isoleucine, leucine, proline, threonine, tryptophan, aspartic acid, lysine and tyrosine, using 20% (V/V) of organic solvent.

In **Figure 36**, it is possible to verify that the lack of efficiency in the separation of the amino acids can lead to a close proximity in the retention times. As in the situation of the elution with 80% (V/V) of ACN, the molecules are in their cationic form, which results in an ionic interaction with the stationary phase and, thus, a retention time very similar among the amino acids. Nevertheless, there is one exception for tryptophan, which is an aromatic amino acid. In this case, the retention time is higher than the others, which can indicate the possibility of distinguishing the tryptophan from the other amino acids in the mixture. There is also another aromatic amino acid being eluted, the tyrosine, but there is a difference in the way tryptophan and tyrosine interact with the aqueous mobile phase. Tryptophan is considered nonpolar while tyrosine is a polar molecule. Besides, tryptophan has an indole group, while tyrosine has a phenol group, which can be the reason why tyrosine is less retained than tryptophan: the phenol group is more prone to be shifted towards the aqueous mobile phase than the indole group, leading to a less interaction between tyrosine and the column stationary phase, and therefore, to a short retention time. Taking into consideration the fact that, in both elutions, with 80% (V/V) and 20% (V/V) of ACN, the amino acids had a similar elution behavior in the Acclaim® Mixed-Mode HILIC-1 stationary phase, another column, the Phenomenex® Luna HILIC, was tested in order to achieve a better separation of these analytes.

4. Results of the preliminary tests on the Phenomenex® Luna HILIC

In this study, the individual amino acids were dissolved in the mobile phase, composed by the following eluents: 15% (V/V) of 5 Mm ammonium acetate (acidified to pH 4.00 with acetic acid), 76.5% (V/V) of ACN, and 8.5% (V/V) of acidified water (ultra-pure water acidified to pH 3.61 with 0.1% (V/V) formic acid). These conditions were kept constant as the ones in **Table 14**. This stationary phase operates mainly in the HILIC mode and the elution is affected by the organic solvent content, since the interactions are related to the polarity of the analytes. The elution conditions were based on the work of Buiarelli *et al.* (2013).

Table 14 Conditions of the preliminary tests for the Phenomenex® Luna HILIC column in order to study the stability of the samples of the aspartic acid, lysine, glycine and tryptophan.

Conditions of the chromatographic separation

Buffer pH:	4.00	Oven temperature:	30ºC
Buffer composition:	Ammonium acetate and acetic acid, 5mM	Flow rate:	0.500 mL/min
Aqueous eluent:	Water acidified with formic acid (pH 3.61)	Organic solvent:	ACN

In this column, and considering the suspicion of degradation of some samples in previous experiments, four different amino acids were eluted one hour apart, in order to study the influence of the time range, since the sample preparation until the injection, in the elution of the sample. A study of the influence of the sonication bath in the elution of the amino acids was also intended. Thus, the chromatograms of samples that were taken to the sonication bath were compared to the ones from samples that were not.

4.1 Influence of the time – from the sample preparation until the injection – and the sonication bath in the stability of an acidic amino acid: aspartic acid

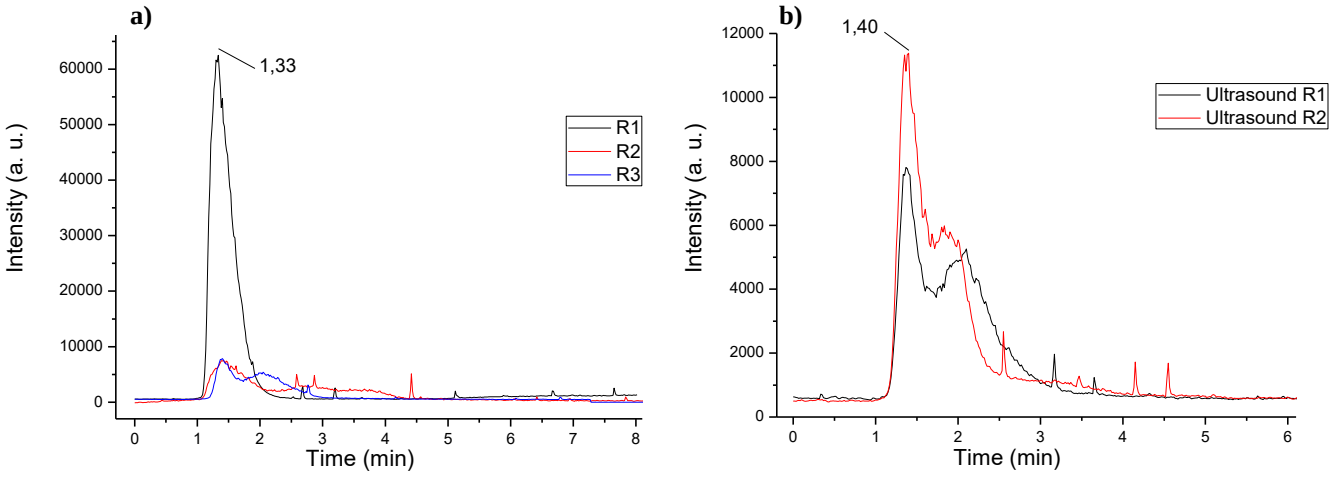


Figure 37 Chromatograms of aspartic acid before (**a)**) and after (**b)**) the sonication bath.

For the aspartic acid, the results show that the retention time remains approximately the same, whether or not the samples were subjected to sonication. In

Figure 37 – a), the retention time remains approximately the same, although the change in the shape and intensity of the chromatographic peaks. The first replica reveals an intense and sharp peak. However, one and two hours later, the result was different from the previous replica: the chromatographic peak was broad and with low intensity. The most probable scenario is the degradation of the amino acid. In **Figure 37 – b)**, the peaks have similar profiles regarding their shape and retention time, although the peak from the second replica is more intense, which indicates the possibility of the evaporation of the solvent. According to the **Figure 37**, the samples are more stable when taken to the sonication bath.

4.2 Influence of the time – from the sample preparation until the injection – and the sonication bath in the stability of a neutral amino acid: glycine

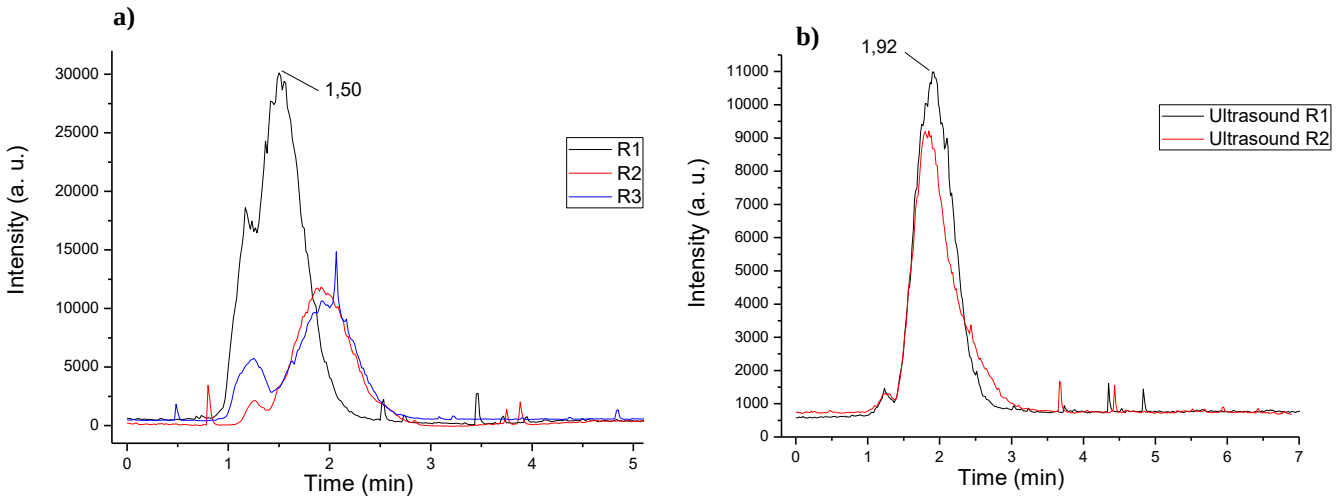


Figure 38 Chromatograms of glycine before (**a)**) and after (**b)**) the sonication bath.

For glycine, there is a significant difference in the stability of the samples when they are treated (**Figure 38 – a**)) with the sonication bath or not (**Figure 38 – b)**). This may imply that, when not taken to the sonication bath, **Figure 38 – a)**, and after some time, glycine suffers degradation that results in the change of the shape, retention time and intensity of the peak. In **Figure 38 – b)**, and after sonication, the chromatographic peaks of the two replicas are very similar: the peaks are analogous regarding not only the

retention time but also their shape and intensity. Considering the **Figure 38**, the peaks of glycine become sharper and more similar to each other after the sonication bath, revealing that the samples are more stable when taken to the sonication bath.

4.3 Influence of the time – from the sample preparation until the injection – and the sonication bath in the stability of a basic amino acid: lysine

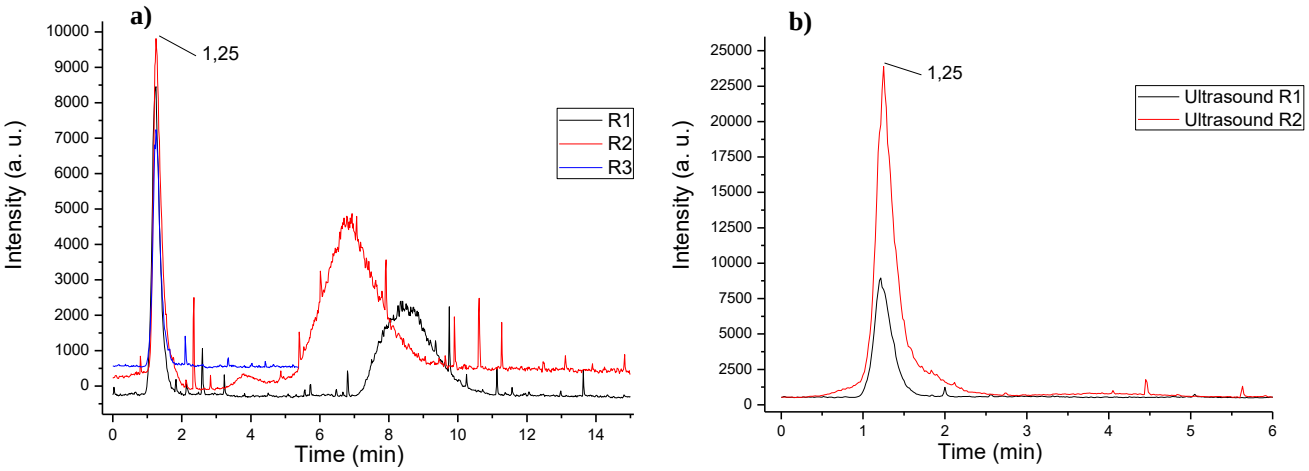


Figure 39 Chromatograms of lysine before (**a)**) and after (**b)**) the sonication bath.

As it is possible to observe in **Figures 39 – a)** and **b)**, there is a stability regarding the retention time (1.25min) of the highest peak in both chromatograms. In **Figure 39 – a)**, although the retention time (1.25min) is the same in the three replicas, the intensity of the signal increases from the first to the second replica and it decreases from the second to the third one. Besides, it seems there was a contamination (the vial could have not been properly clean) in the first two elutions. However, the retention time of the broader peak decreases and its' intensity increases from the first to the second elution. In **Figure 39 – b)**, after the sonication, the peaks became sharper and the interferences from the chromatograms in **Figure 39 – a)** disappeared, indicating that those interferences may have resulted from lysine particles that were not totally dissolved in the solution. Furthermore, the value of the retention time remained the same between the two elutions, despite of the increase of the intensity of the signal, showing some stability in the process

over time. Considering the **Figure 39**, the peaks in the chromatograms of lysine show a more stable behavior when the sample is taken to the sonication bath.

4.4 Influence of the time – from the sample preparation until the injection – and the sonication bath in the stability of an aromatic amino acid: tryptophan

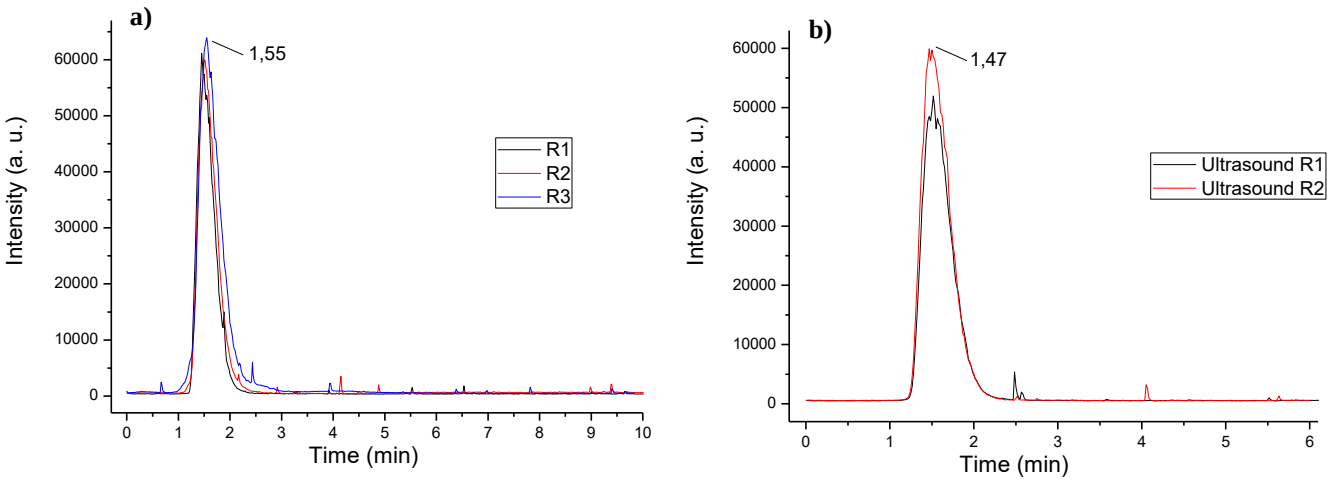


Figure 40 Chromatograms of tryptophan before (**a)**) and after (**b)**) the sonication bath

The tryptophan revealed to be the most stable one among the studied amino acids. In this case, even the replicas that were not taken to the sonication were reproducible, both regarding the retention time, the peak intensity and shape, as shown in **Figure 41 – a)** and **b)**. In **Figure 40 – b)**, in which the samples were taken to the sonication before they were injected, the obtained results revealed a reproducibility regarding the retention time but not concerning the intensity, which increased from the first to the second replica.

Considering all the results, it is possible to conclude the lack of stability over time of the samples (aspartic acid, glycine and lysine) that were not taken to the sonication bath, which indicates that the samples must be prepared and injected right after the dissolution or be taken to the sonication bath. In these conditions, the samples showed some stability regarding both retention and peak shape. Moreover, when comparing the

retention of the same amino acid but in the different conditions, it is possible to observe a slight difference in the values of the retention time. Nevertheless, the retention times of all the amino acids being studied are close to each other, which does not guarantee the success in distinguishing them in a mixture with all the 20 amino acids.

5. Results of the preliminary tests on the Phenomenex® Luna C18

5.1 Gradient selection for the chromatographic separation

In this column, the 20 individual amino acids were dissolved, in equal concentrations (concentration of each amino acid in the 20 amino acids mixture: 0.050 mM), in acidified water (pH = 3.00, adjusted with formic acid), and eluted in gradient mode, based on the results of Bullock (2008). A few differences were implemented comparing to the authors work: the temperature of the oven (30º C) and the flow rate (1.000 mL/min). The conditions that were kept constant are shown in **Table 15**. From the initial gradient suggested by Bullock (2008), **Figure 41**, other gradients were studied in order to improve the separation of the amino acids in the mixture, according to the influence of the organic solvent content in the mobile phase and respective elution time. In **Table 17**, at the end of the chapter, all the gradients that were used are shown.

Table 15 Conditions of the preliminary tests for the Phenomenex® Luna C18 column applied in the selection of the gradient mode which separates more amino acids.

Conditions of the chromatographic separation

pH:	3.00	Oven temperature:	30ºC
Aqueous eluent:	Water acidified with formic acid	Flow rate:	1.000 mL/min
Organic solvent:	ACN		

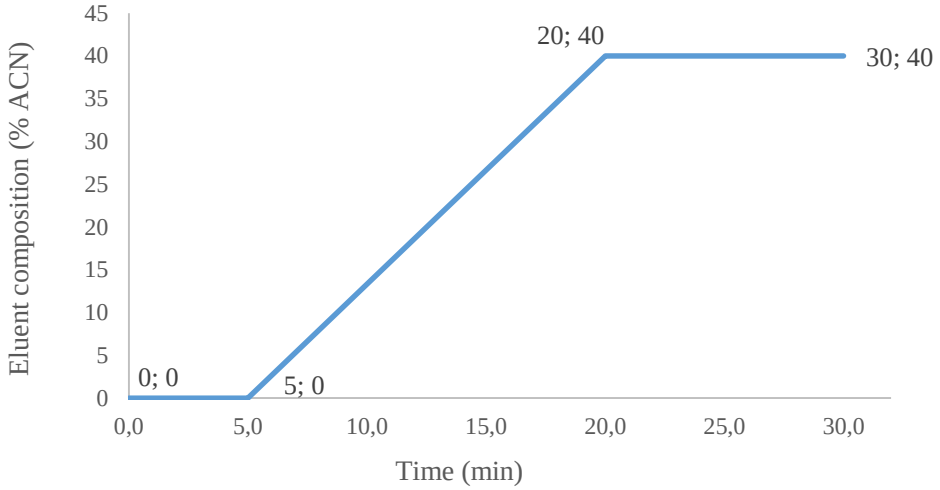


Figure 41 Gradient 1 applied to elute the 20 amino acids mixture.

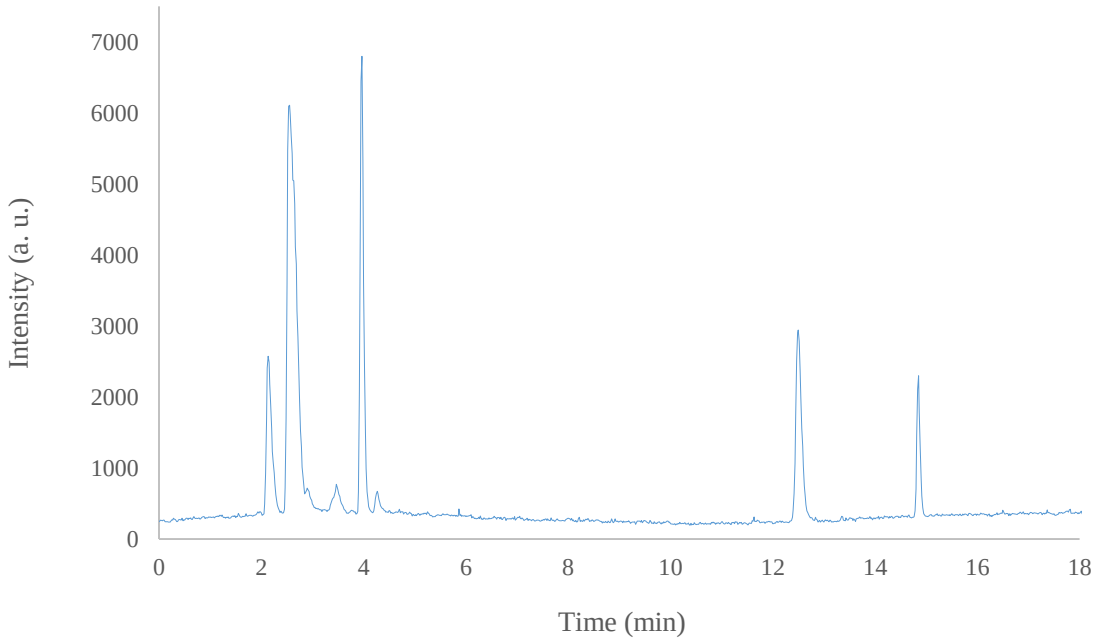


Figure 42 Chromatogram obtained with the gradient 1 showing seven peaks in the separation of the 20 amino acids mixture.

With the gradient shown in **Figure 41**, it was possible to obtain seven distinct peaks (**Figure 42**) out of twenty amino acids in the mixture. Thus, in order to improve the separation method and to understand the influence of the organic solvent in the elution of the mixture, another gradient was tried (**Figure 43**).

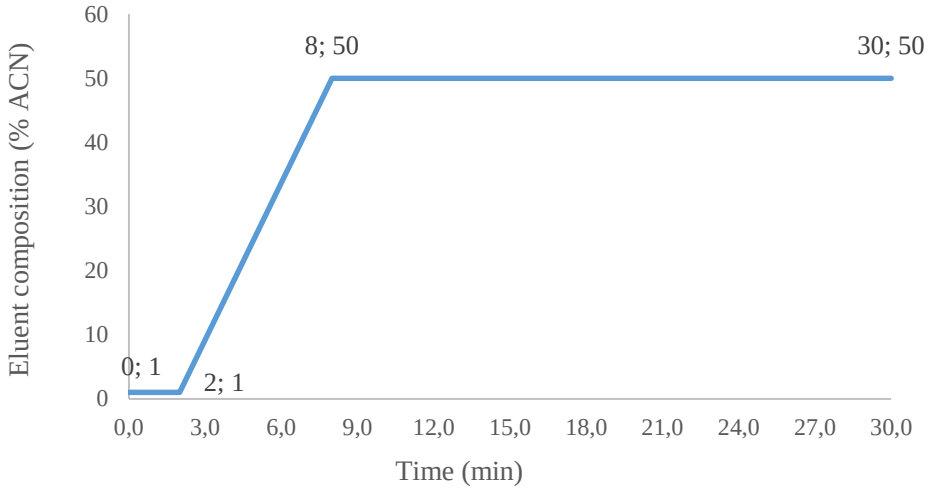


Figure 43 Gradient 2 applied to elute the 20 amino acids mixture.

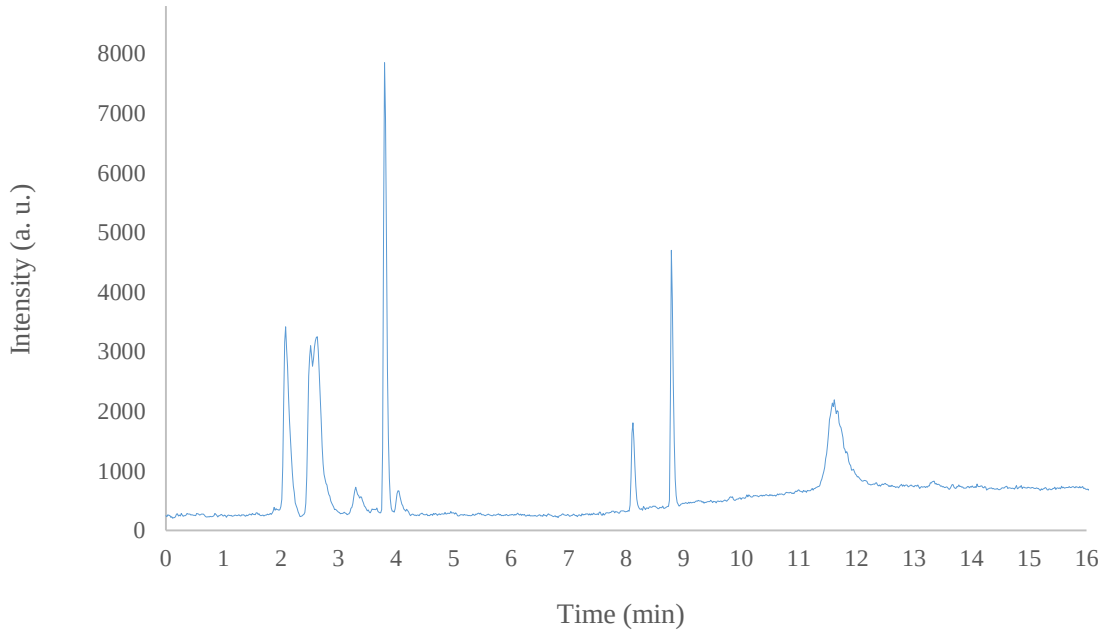


Figure 44 Chromatogram obtained for the gradient 2, showing eight different peaks in the separation of the 20 amino acids mixture.

Using this second gradient (**Figure 43**), the chromatogram of **Figure 44** shows more peaks (eight), all eluted before 12 minutes. The new gradient started with 1% (V/V) of ACN for only 2 minutes, and increased to 50% (V/V) of ACN during 6 minutes, resulting in more compounds being separated and in a faster elution. Thus, the third gradient started with a higher amount of organic solvent, followed by an increase to 5%

(V/V) of ACN during 5 minutes. After that, the elution was made with 5% (V/V) of ACN for 13 minutes, having another fast rise from the 5 to 25% (V/V) of ACN in 5 minutes, as shown in **Figure 45**.

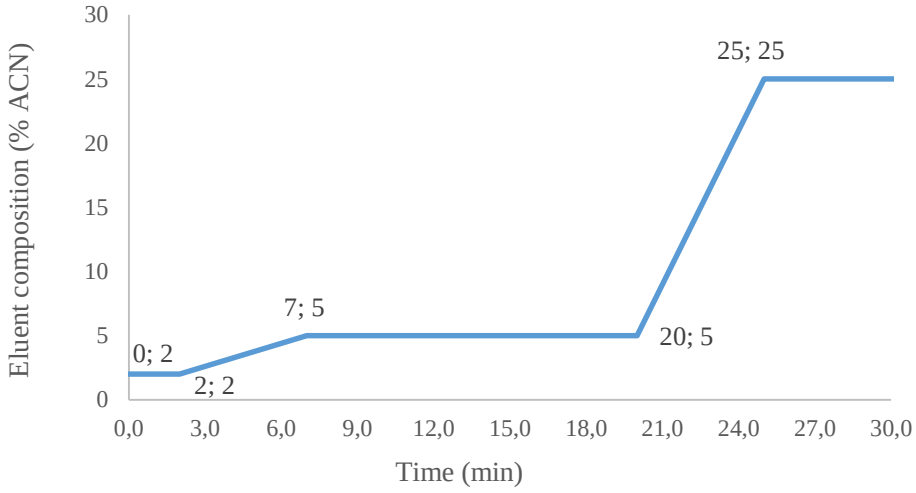


Figure 45 Gradient 3 applied to elute the 20 amino acids mixture.

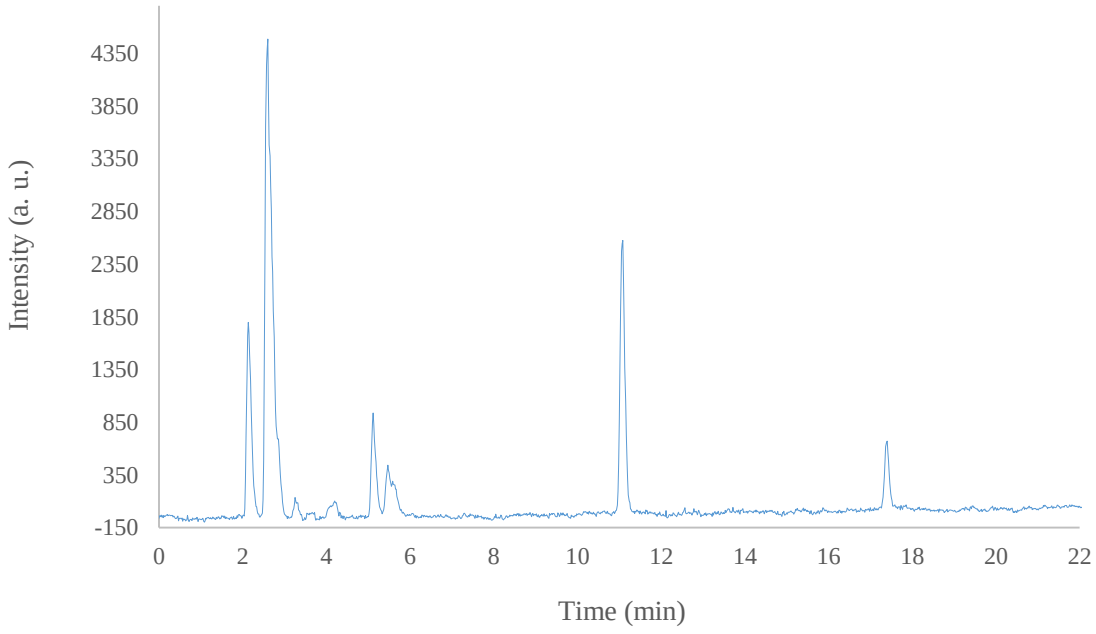


Figure 46 Obtained results from the gradient 3, with eight distinct peaks in the elution of the mixture with 20 amino acids.

As observed in **Figure 46**, more compounds are eluted comparing to the gradients 1 and 2. Nevertheless, the peaks from 2 to 4 minutes remained with the same retention,

although their intensity and shape are very different when compared to the previous results. Besides, the last peak appeared around 18 minutes, indicating there was an increase of the retention time.

Taking into account that the elution may be more effective with more short levels of isocratic mode with low values of the organic solvent content, the gradient 4 was created and is shown in **Figure 47**.

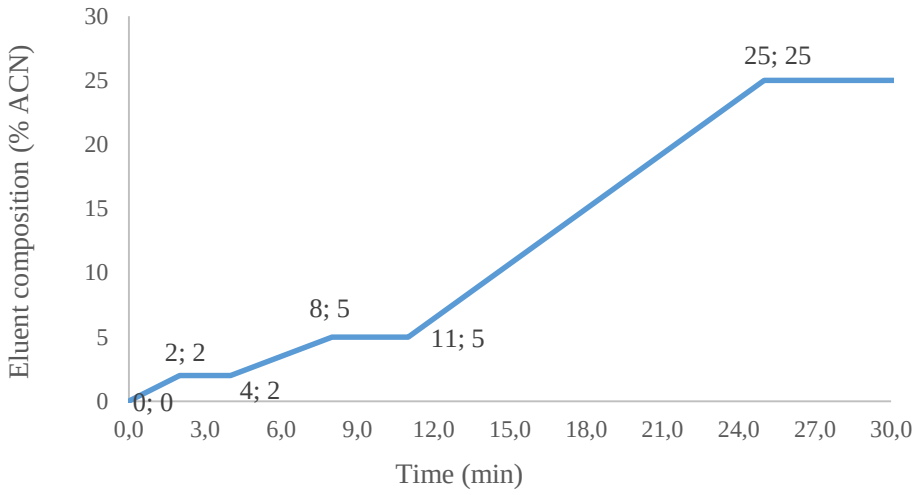


Figure 47 Gradient 4 applied to elute the 20 amino acids mixture.

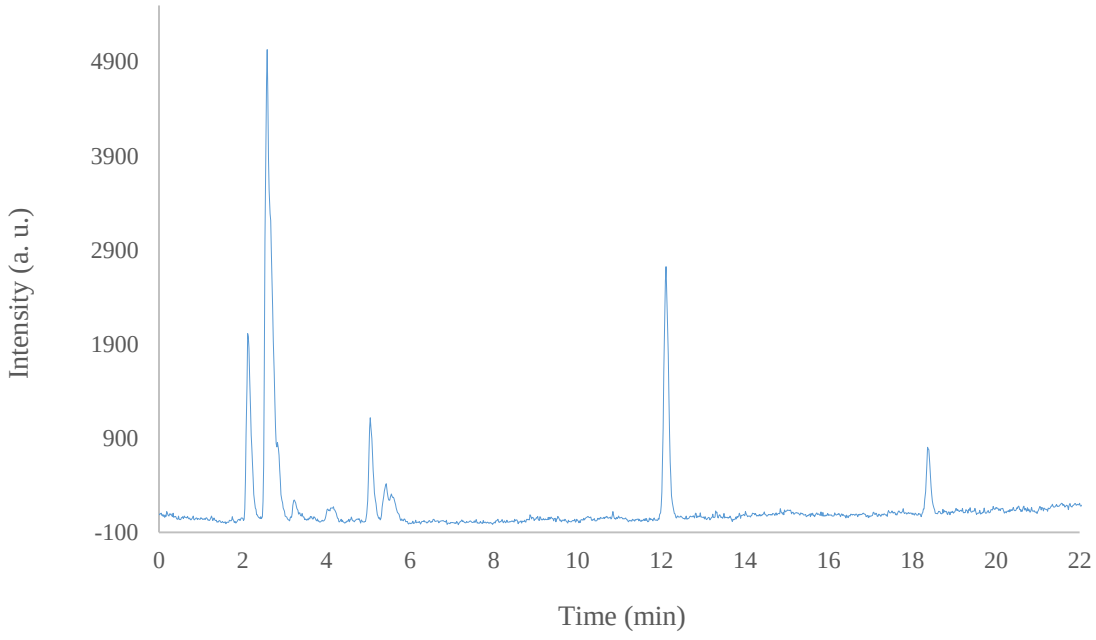


Figure 48 Chromatogram obtained for the gradient 4, showing eight different peaks in the separation of the 20 amino acids mixture.

In **Figure 48**, the number of peaks are less. Besides, the total of the elution time slightly increased, which suggests that the amount of isocratic levels and/or the slope in the gradient program may have an effect on the retention of the compounds in the mixture. In an attempt to obtain better results, gradient 5 was applied, **Figure 49**.

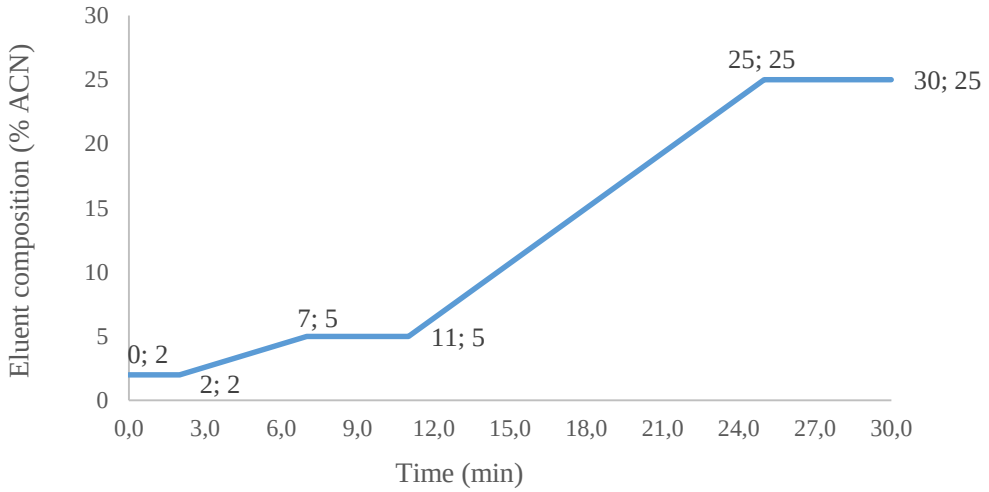


Figure 49 Gradient 5 applied to elute the 20 amino acids mixture.

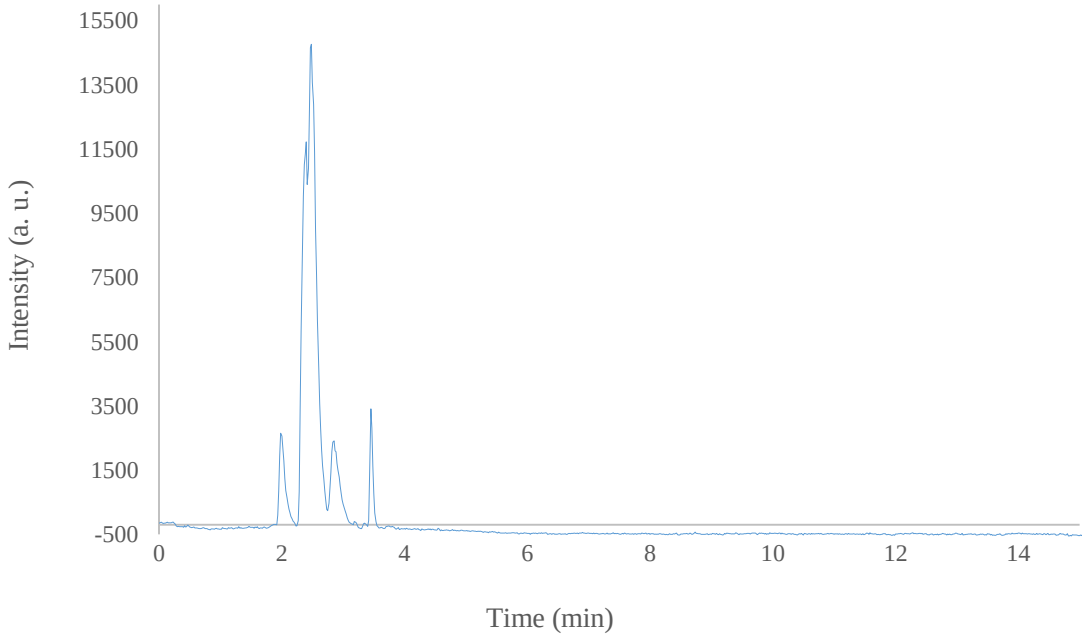


Figure 50 Chromatogram obtained for the gradient 5, showing four different peaks in the separation of the 20 amino acids mixture.

With gradient 5, the chromatogram had less number of peaks, only four, and they all appeared between the 2 and 4 minutes, as shown in **Figure 50**. Since with this experiment, the 20 amino acids were not efficiently separated, gradient 6 was designed based on a slower increase of the organic solvent content from the first to the second level and a faster one from the second to the third level, as shown in **Figure 51**.

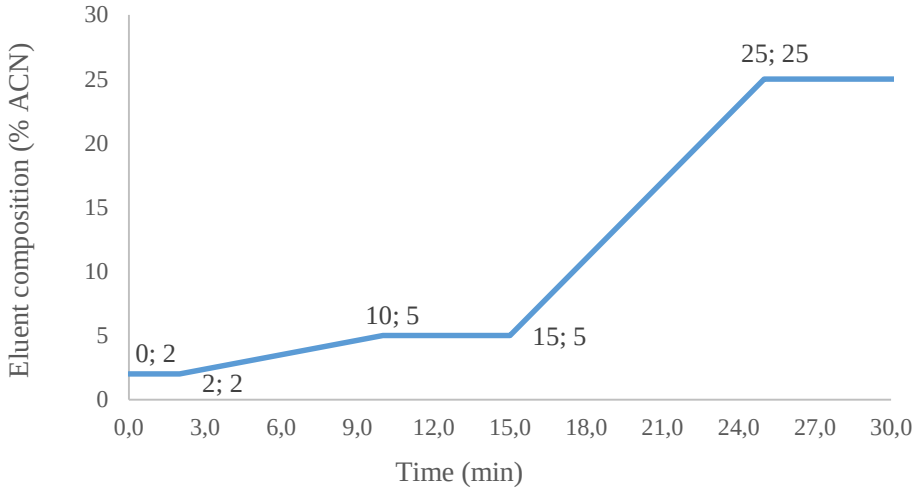


Figure 51 Gradient 6 applied to separate the 20 amino acids in the mixture.

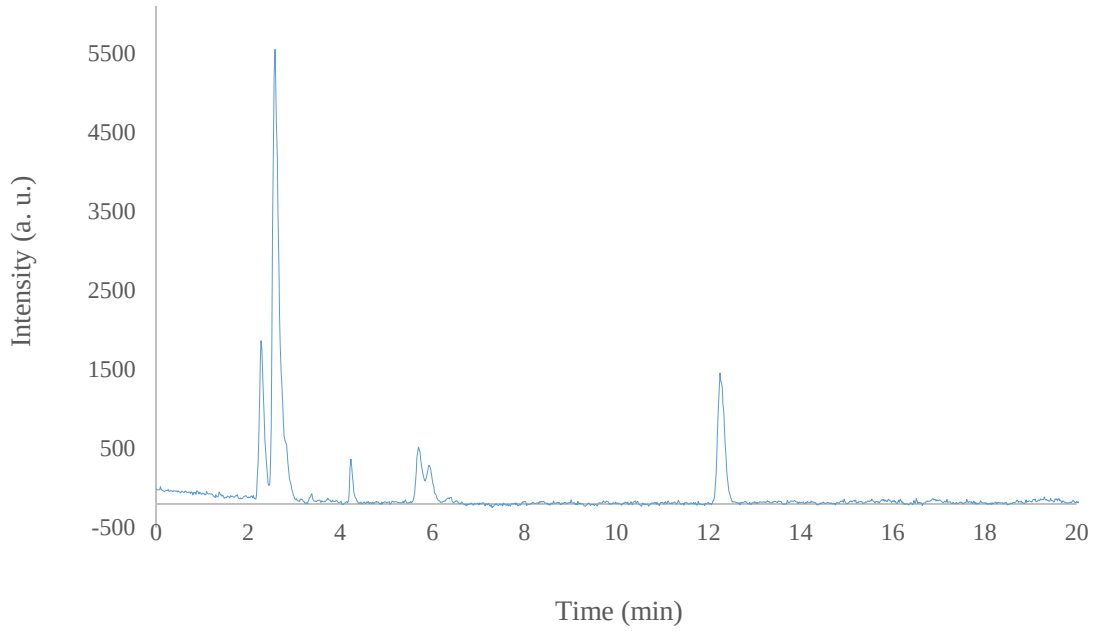


Figure 52 Chromatogram obtained for the gradient 6, showing five different peaks in the separation of the 20 amino acids mixture.

The results with gradient 6 did not improved, since only five peaks were obtained (**Figure 52**). Hereafter, the initial step of the gradient was kept the same and the difference occurred in the duration of the second isocratic moment of the elution: the isocratic mode lasted longer and the increase of the amount of acetonitrile was faster from the second to the third level, as shown in **Figure 53**.

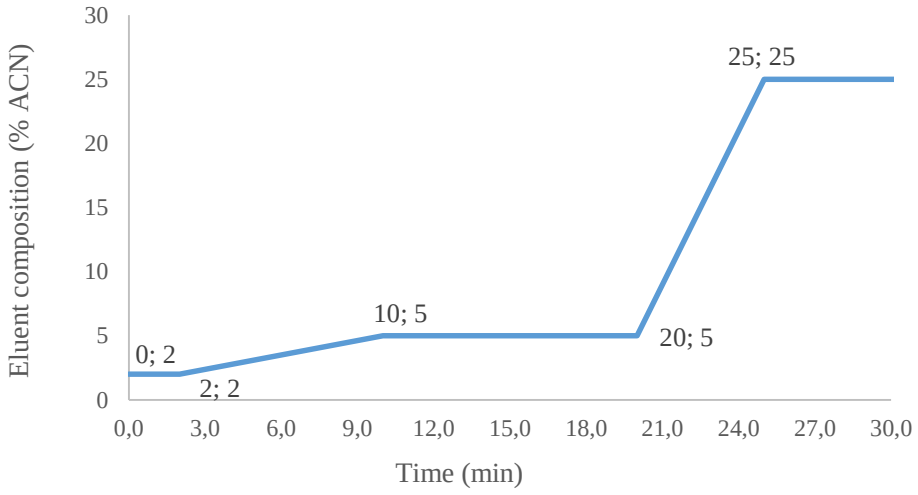


Figure 53 Gradient 7 applied to separate the 20 amino acids in the mixture.

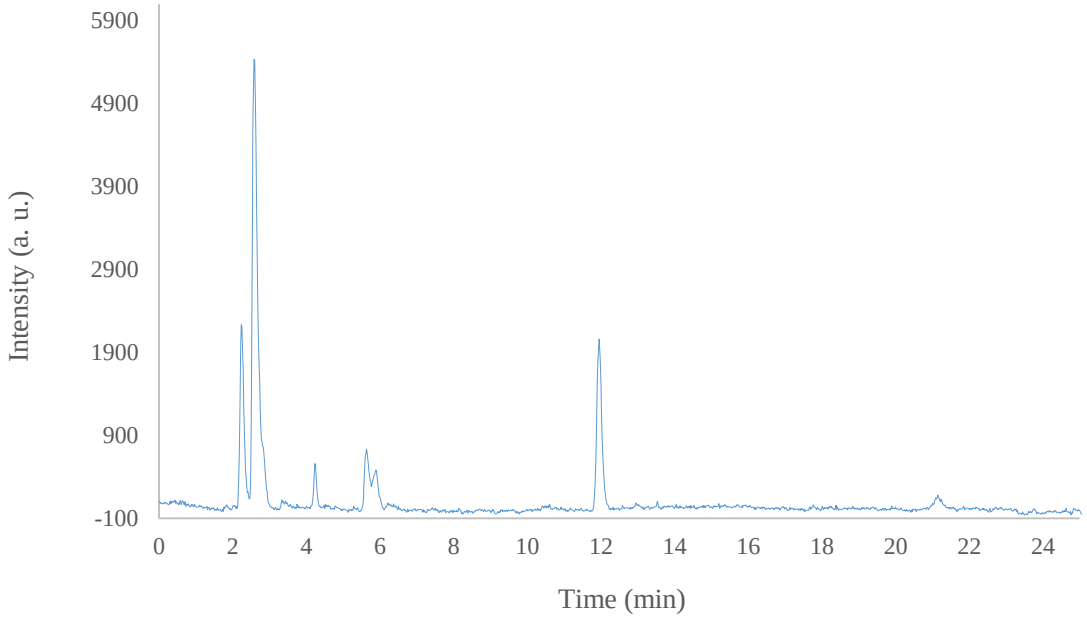


Figure 54 Chromatogram obtained for the gradient 7, showing six different peaks in the separation of the 20 amino acids mixture.

The chromatogram in **Figure 54**, revealed deeply similar results both in number of different peaks as in their retention time, only with a different profile: there was a small wide peak at approximately 20.5 minutes. Although there was a slight improvement in the number of distinguished peaks from five to six, it was not enough regarding the main goal for the separation. Therefore, and considering that a faster increase of the ACN content was not leading to a better separation, a reverse gradient was experimented: start with a small amount of organic solvent, decrease it for a few minutes, and increase it again until the end of the elution, as shown in **Figure 55**.

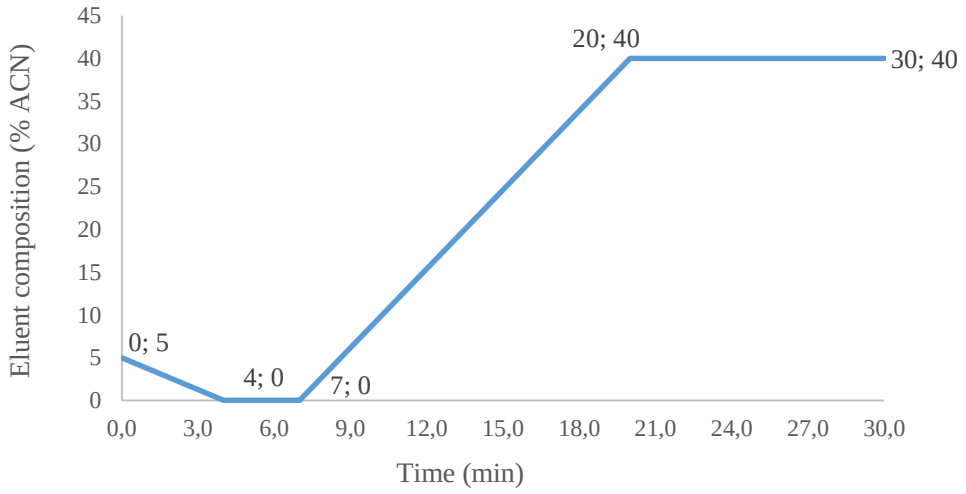


Figure 55 Gradient 8 applied to separate the 20 amino acids in the mixture.

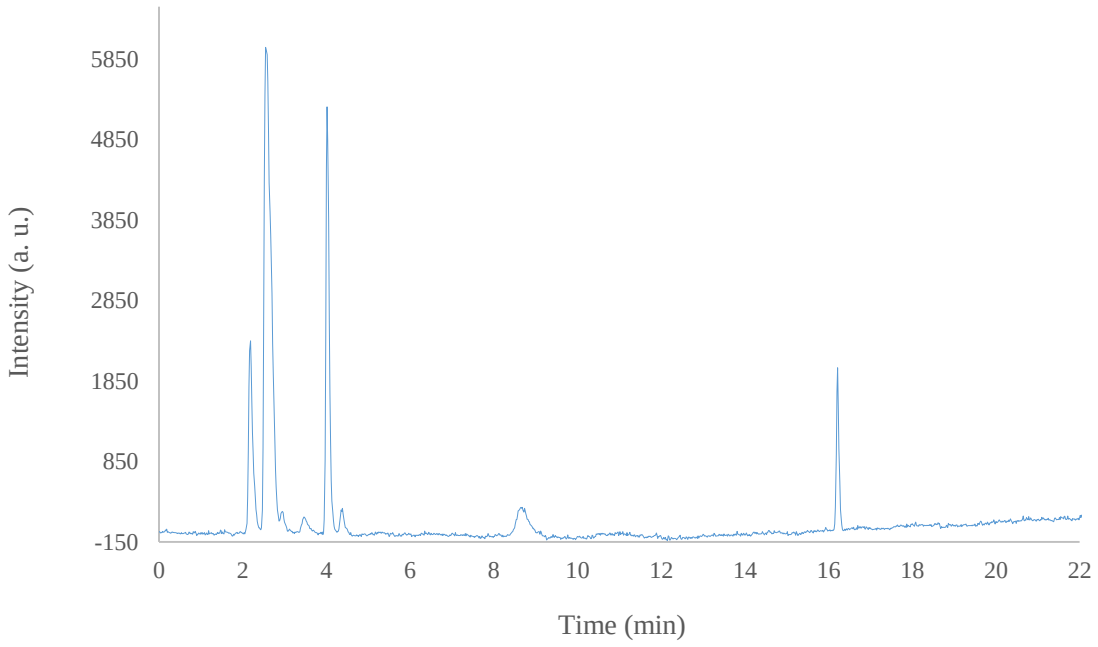


Figure 56 Chromatogram obtained for the gradient 8, showing seven different peaks in the separation of the 20 amino acids mixture.

An improvement was made regarding the separation of the amino acids, since seven well defined peaks were eluted (**Figure 56**). Therefore, starting with a small amount of organic solvent, decrease it to zero and increase it again seems to help in the separation of the compounds in the synthetic mixture. Thus, the same principle was applied but with a shorter duration: instead of 4 min, the decrease of the organic solvent concentration takes 3 min from 5% (V/V) to zero (**Figure 57**).

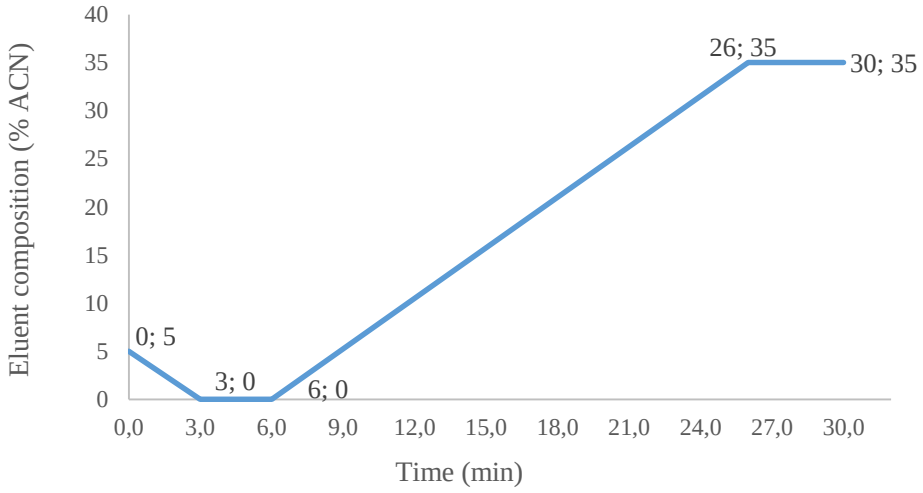


Figure 57 Gradient 9 applied to separate the 20 amino acids in the mixture.

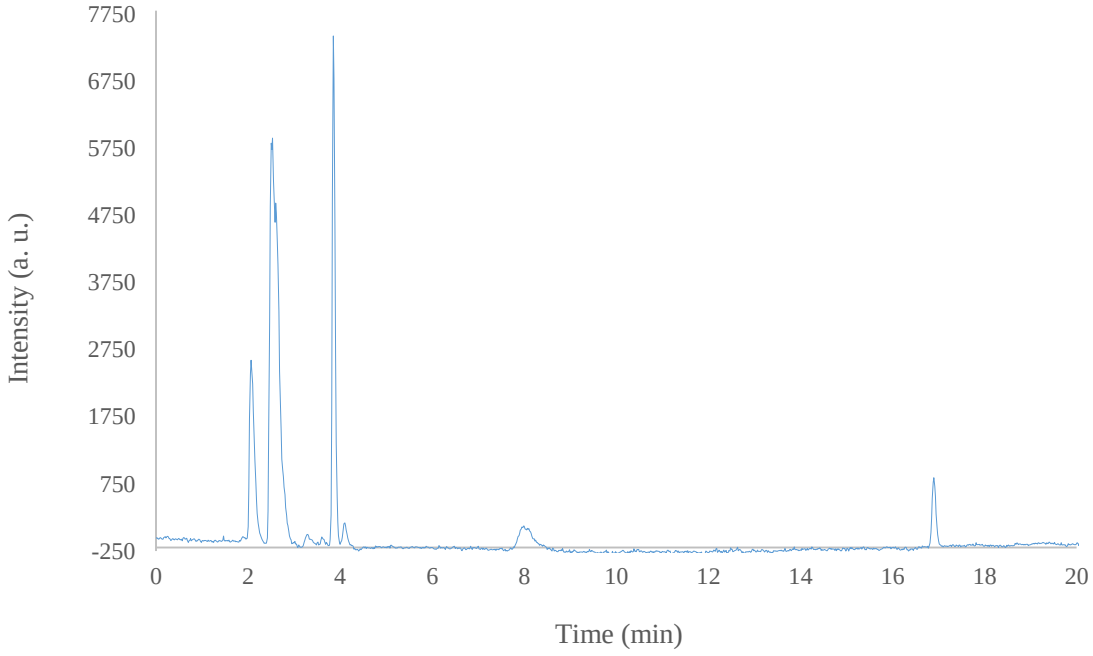


Figure 58 Chromatogram obtained for the gradient 9, showing eight distinct peaks in the separation of the 20 amino acids mixture.

The chromatogram in **Figure 58** reveals that eight compounds were successfully separated, which means a slight improvement of the process regarding the number of peaks obtained with the gradient 8 (**Figure 56**). Thus, in order to further improve the separation of the studied amino acids, a different a gradient was assessed, starting from a small amount of organic solvent, followed by a slow decrease of its content, then by an isocratic elution for a 8min and finally two different increases of the organic solvent concentration at distinct speeds: from 2% (V/V) to 20% (V/V) in 4min and from 20% (V/V) to 35% (V/V) in 15min (**Figure 59**).

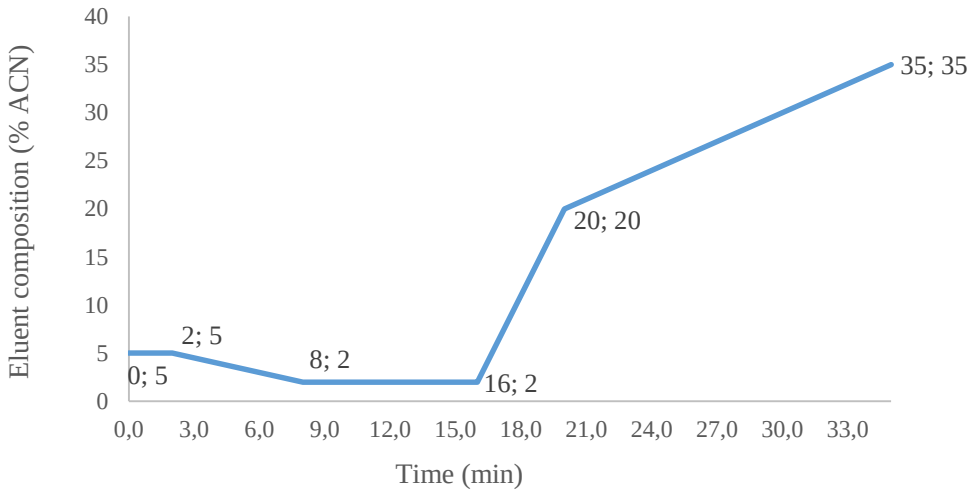


Figure 59 Gradient 10 applied to separate the 20 amino acids in the mixture.

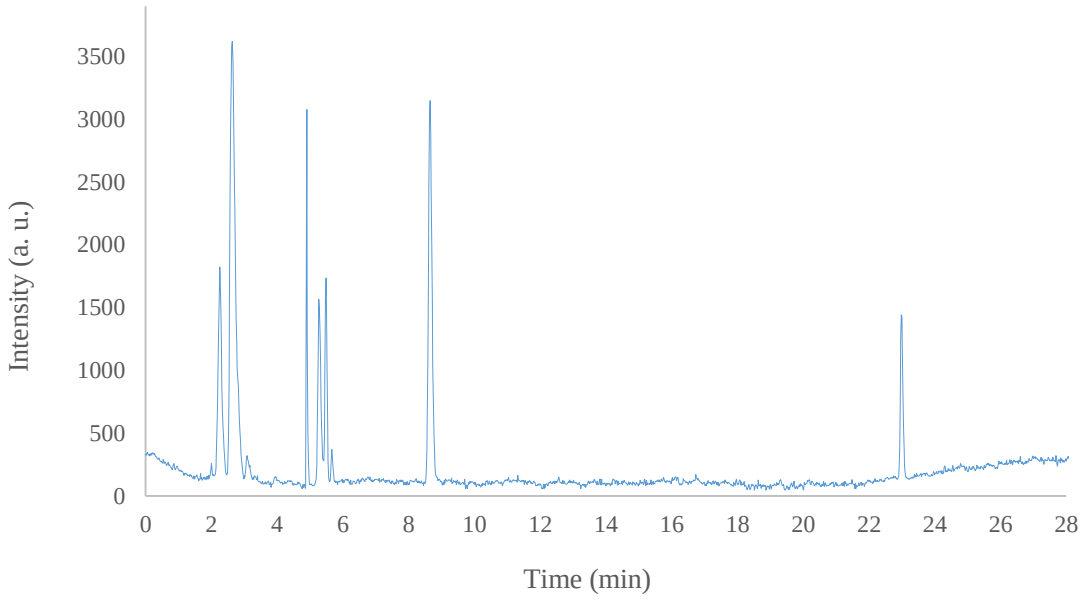


Figure 60 Chromatogram obtained for the gradient 10, showing ten different peaks in the separation of the 20 amino acids mixture.

The improvement of the separation process is notable in the chromatogram in **Figure 60**. The results showed ten distinct and sharp peaks, being the best gradient obtained in the experiments. Thus, this gradient was chosen to be used for the chromatographic separation of the aqueous extracts from each collected bulk aerosol samples. Furthermore, each individual amino acid was also eluted in gradient 10, in order to know which amino acids corresponds to which peak. The chromatogram with all the twenty amino acids eluted individually is shown in **Figure 61** and their retention times are shown in **Table 16**.

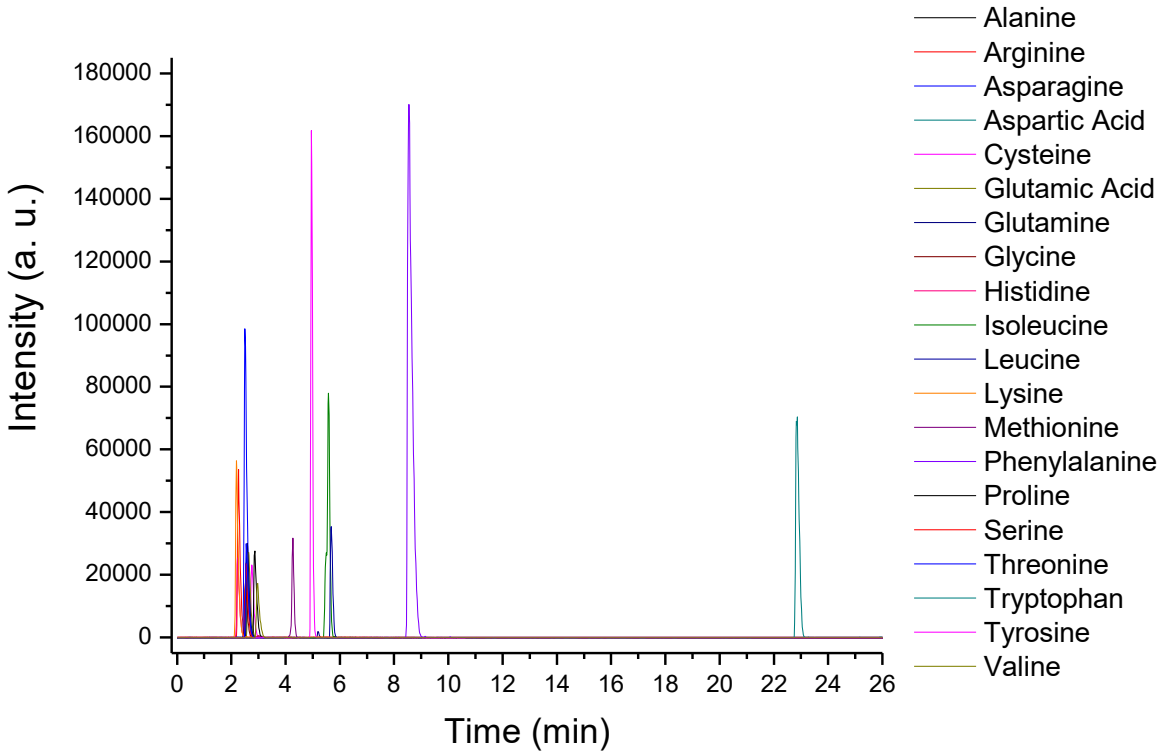


Figure 61 Chromatogram with all the twenty amino acids eluted individually.

Table 16 Amino acids and their respective retention times with gradient number 10.

Amino acid	Retention time (min)	Amino acid	Retention time (min)
Lysine	2.183	Glutamic acid	2.633
Histidine	2.233	Cysteine	2.750
Arginine	2.267	Proline	2.867
Asparagine	2.500	Valine	2.967
Glycine	2.517	Methionine	4.267
Serine	2.533	Tyrosine	4.950
Threonine	2.550	Isoleucine	5.583

Alanine	2.557	Leucine	5.683
Glutamine	2.583	Phenylalanine	8.550
Aspartic acid	2.617	Tryptophan	22.867

Using the information from both the chromatogram in **Figure 61** and **Table 16**, it was possible to assign the amino acids to the corresponding chromatographic peak. The match is shown in **Figure 62**.

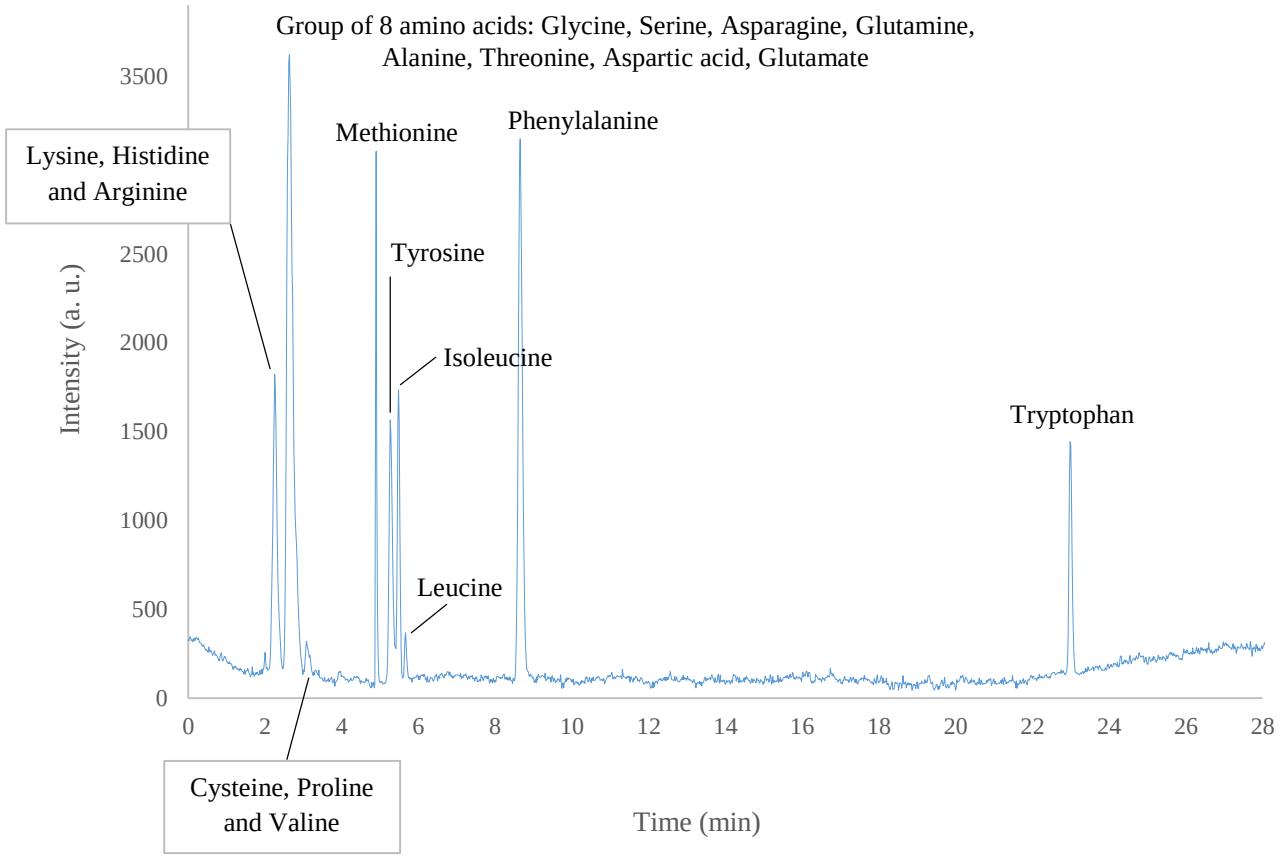


Figure 62 Matching of the results obtained for the mixture and the individual results.

All the gradients that were used for the chromatographic separation of the amino acids in the mixture are shown in **Table 17**.

Table 17 Programs of the 10 gradients used in the improvement of the chromatographic separation of amino acids. The percentages represent the concentration of the ACN in the mobile phase.

Gradient 1	0 – 5 min 0 %	5 – 20 min 0 – 40 %	20 – 30 min 40 %			
Gradient 2	0 – 2 min 1%	2 – 8 min 1 – 50 %	8 – 30 min 50%			
Gradient 3	0 – 2 min 2%	2 – 7 min 2 – 5 %	7 – 20 min 5%	20 – 25 min 5 – 25 %	25 – 30 min 25%	
Gradient 4	0 – 2 min 0 – 2 %	2 – 4 min 2%	4 – 8 min 2 – 5 %	8 – 11 min 5%	11 – 25 min 5 – 25 %	25 – 30 min 25%
Gradient 5	0 – 2 min 2%	2 – 7 min 2 – 5 %	7 – 11 min 5%	11 – 25 min 5 – 25 %	25 – 30 min 25%	
Gradient 6	0 – 2 min 2%	2 – 10 min 2 – 5 %	10 – 15 min 5%	15 – 25 min 5 – 25%	25 – 30 min 25%	
Gradient 7	0 – 2 min 2%	2 – 10 min 2 – 5 %	10 – 20 min 5%	20 – 25 min 5 – 25 %	25 – 30 min 25%	
Gradient 8	0 – 4 min 5 – 0 %	4 – 7 min 0%	7 – 20 min 0 – 40 %	20 – 30 min 40%		
Gradient 9	0 – 3 min 5 – 0 %	3 – 6 min 0%	6 – 26 min 0 – 35 %	26 – 30 min 35%		
Gradient 10	0 – 2 min 5%	2 – 8 min 5 – 2 %	8 – 16 min 2%	16 – 20 min 2 – 20 %	20 – 35 min 20 – 35 %	

IV

Results and discussion

1. Liquid chromatography separation

The six different collected samples were analyzed via liquid chromatography in order to separate the amino acids from the other particles present in the atmosphere of Aveiro. The elutions were performed with a Phenomenex® Luna C18, at 1.000 mL/min flow and an oven temperature of 30ºC. Although ACN and acidified water (pH = 3.00 adjusted with formic acid) were used as eluents for the mobile phase, the samples were dissolved only in acidified water. The results of the chromatographic separation are shown in **Figure 63**. Samples 1 (bottom) and 2 (up) were collected from the sampler near the Glicínias Plaza mall. Samples 3 (bottom) and 4 (up) were collected from the sampler in the horizontal position at the STIC Department. Samples 5 (bottom) and 6 (up) were extracted from the sampler in the vertical position at the STIC Department.

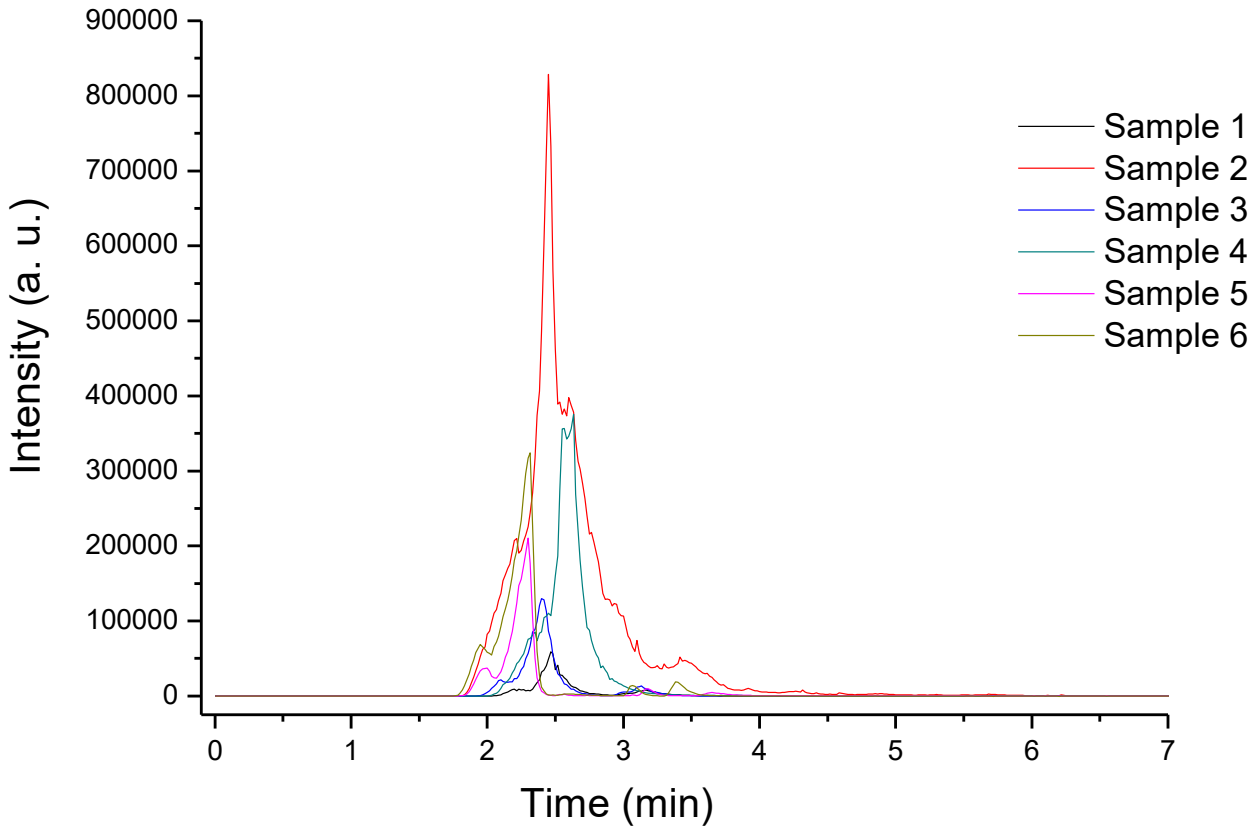


Figure 63 Chromatogram showing the results of the elution of the six different samples collected in two different sites in the city of Aveiro. The aqueous extracts of the bulk aerosol samples were directly injected after the extraction process.

As it is possible to verify in **Figure 63**, the components of the six samples are all eluted in less than 5 minutes in the chosen elution conditions. According to the results obtained in the preliminary tests for the same conditions (**Figure 61** and **Table 16**), fourteen amino acids are eluted between 1.8 and 4 minutes: lysine, histidine, arginine, glycine, serine, asparagine, glutamine, alanine, threonine, aspartic acid, glutamic acid, cysteine, proline and valine. Although eluting within the same range of retention times, it is not possible to guarantee the presence of those fourteen amino acids in the studied aerosol samples. Besides, there is a possibility that compounds other than amino acids could also appear at the same range of retention times. As studied by Heintzenberg (2003), the European urban atmospheres are filled with both organic and inorganic species. According to the author, only around 12-15% of the particles are organic and the inorganic matter is mostly composed by sulfate, nitrate, ammonium, sodium and chloride. Thus, and considering the chromatograms in **Figure 63**, the mentioned particles (sulfate, nitrate, ammonium, sodium and chloride) may also have been collected during the sampling and dissolved in the extraction process. Bearing in mind the location of the samplers and their height from the ground, the existence of inorganic matter is highly probable.

The results in **Figure 64** also show that the filter corresponding the Sample 2 was the filter with higher amount of collected material, and it corresponds to the filter with bird activity. Samples 1 and 3 (bottom filters) have a similar profile, although the intensity is significantly distinct, just like Samples 2 and 4 (up filters). Samples 5 and 6 (bottom and up filters from the samplers in the vertical position) have a similar shape and intensity.

In order to have a better comparison regarding the effect of samplers location, near Glicínias Plaza mall and at the University of Aveiro, as well as the position of the filter, bottom or up, the chromatograms were rearranged according to these features and are shown in the following figures.

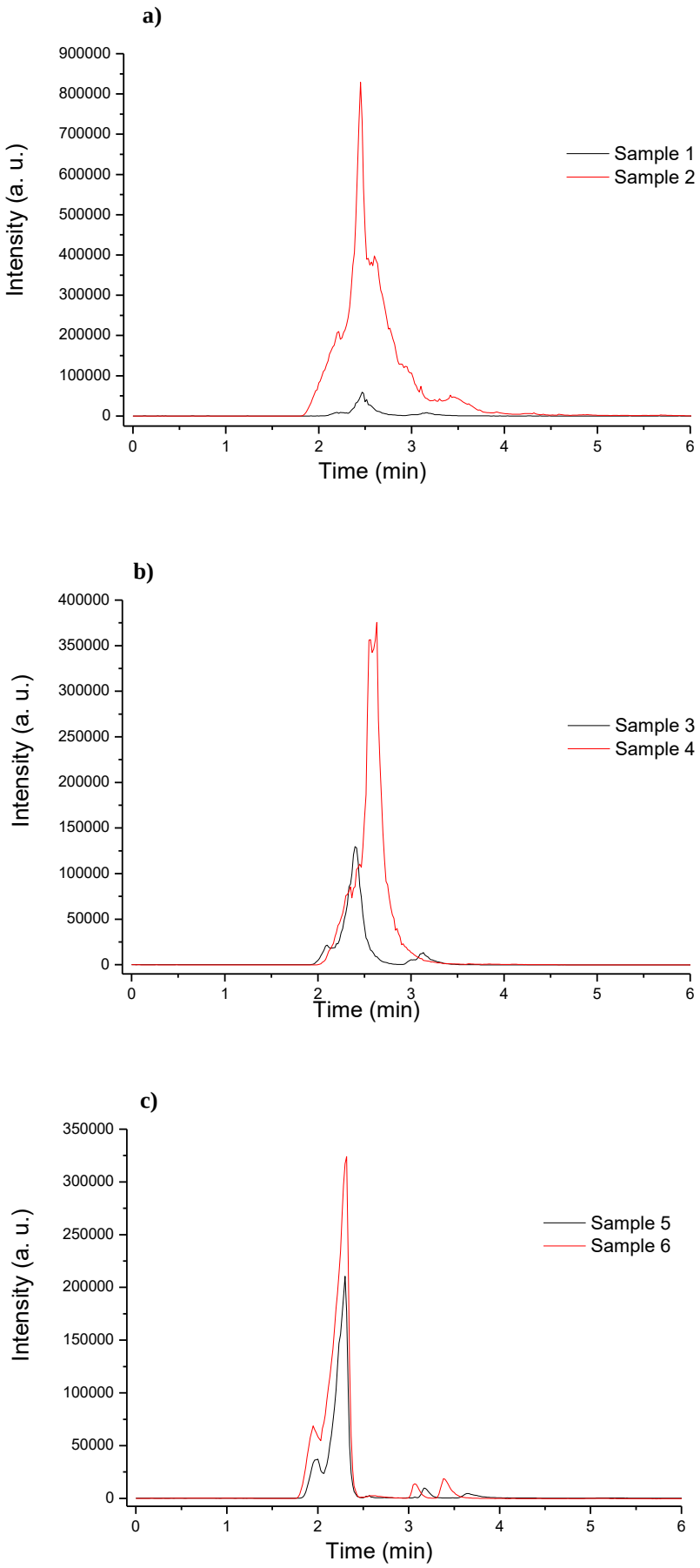


Figure 64 Chromatograms related to the sampler location: **a)** near Glicínias Plaza mall; **b)** and **c)** at the University of Aveiro.

According to the results in **Figure 64 – a)**, both the aqueous extracts have a similar elution behavior, with the most intense chromatographic peak eluting at around 2.5 minutes. The difference in the intensity between the two different samples may be related to their position in the sampler: sample 1 was extracted from the bottom filter and sample 2 from the up filter (containing bird activity).

Chromatograms in **Figure 64 – b)** do not show a similar profile, including also their intensity. Filters 3 and 4 were placed at the roof of the STIC Department of the University of Aveiro and their differences may have resulted from the fact that one was a bottom filter (number 3) and the other was a top filter (number 4). Filter number 3 may have more inorganic matter coming from the ground, while filter number 4 collected much more material, probably both organic and inorganic.

In **Figure 64 – c)**, it is possible to observe that the signals are very similar regarding both the profile and the intensity. Filters 5 and 6 were placed in the sampler that was in a vertical position. They also had the same height from the ground. Considering these factors, the slight differences in the signals may be due to the variation of the wind direction and speed during the sampling days.

In **Figure 65 – a)**, it is possible to notice the close resemblance between the chromatograms. Although the filters were in different locations in Aveiro, they both were bottom filters, which may explain the strong similarity, indicating that the two filters may have collected similar material. The difference in the intensity values may result from the height of the sampler to the ground: filter 1 was in a sampler embedded in a railing of a balcony in the second floor, approximately 7m from the ground, while filter 3 was in a sampler approximately 1.50 meters from the ground (considering the top of the building).

Regarding the top filters, in **Figure 65 – b)**, the results reveal a similar profile with a few differences both in the shape and the intensity of the chromatographic peaks, justified by the location of the samplers: the sampler related to filter number 2 was placed near a construction site, which contributes to an increase of the quantity of the re-suspended material. Besides, filter number 2 was the one with birds' activity solids, which led to an increase of the amount of the collected material in the filter.

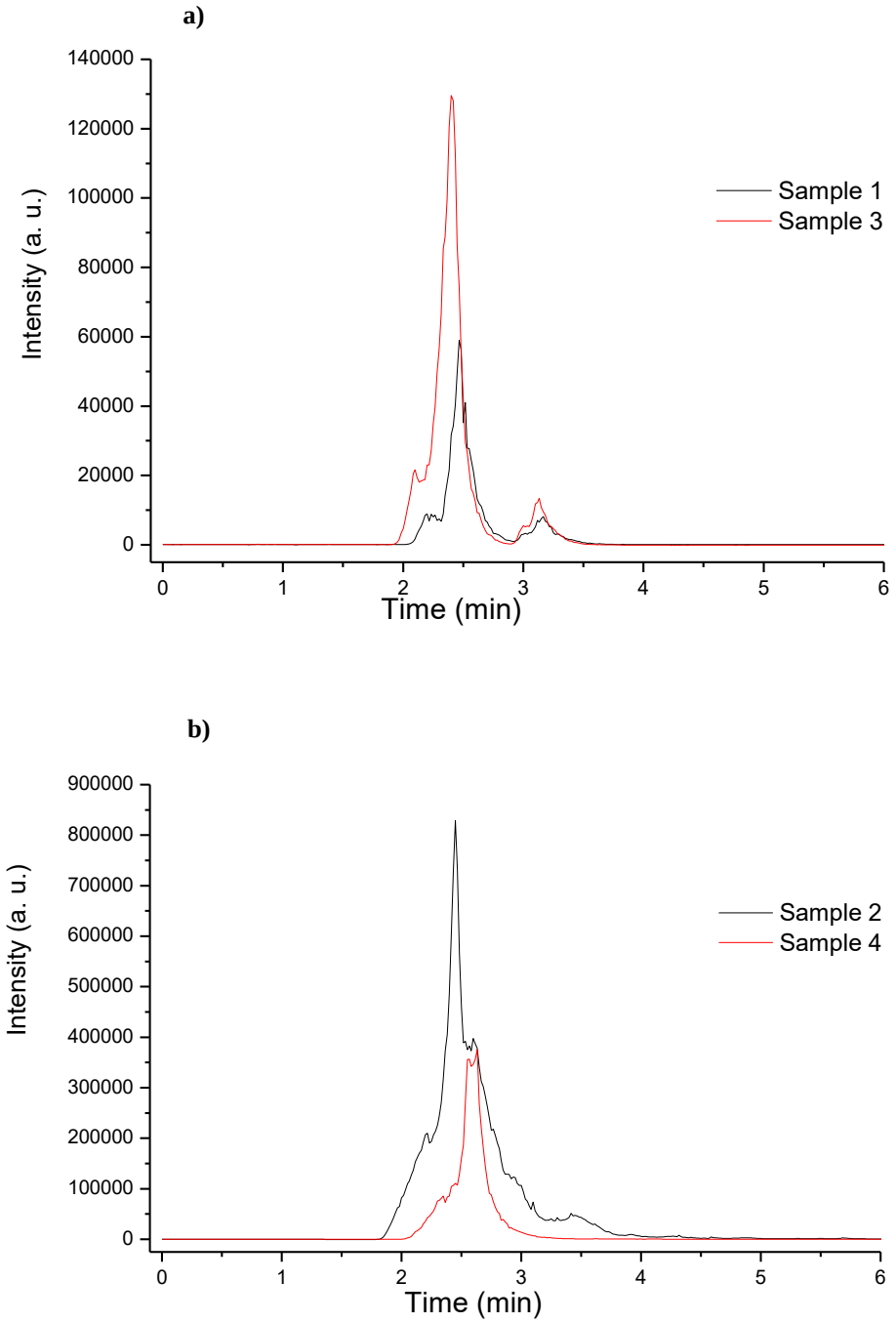


Figure 65 Chromatograms regarding the position of the filters relatively to the samplers: **a)** bottom filters; **b)** up filters. Sample 1 was extracted from filter 1; sample 2 was extracted from filter 2; sample 3 was extracted from filter 3; and sample 4 was extracted from filter 4.

2. Excitation-emission matrix fluorescence spectroscopy analyzes

The main fluorophores structures of the aqueous extracts of the bulk aerosol samples were also studied by three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy. The samples were diluted in acidified water (Sample 1 – 5x, Sample 2 – 100x, Sample 3 – 10x, Sample 4 – 45x, Sample 5 – 20x, Sample 6 – 30x) in order to have intensity values in a range that the spectrophotometer could detect without showing saturated spectra. Later, those diluted samples were the ones being used for the fluorescence spectroscopy spectra. They were again diluted, in ultra-pure water, in a dilution factor of 10 for the EEM fluorescence spectroscopy. The solvent (ultra-pure water) spectrum was obtained and used as a blank. The EEM fluorescence spectra of the 6 samples are shown below, in **Figures 66** to **71**.

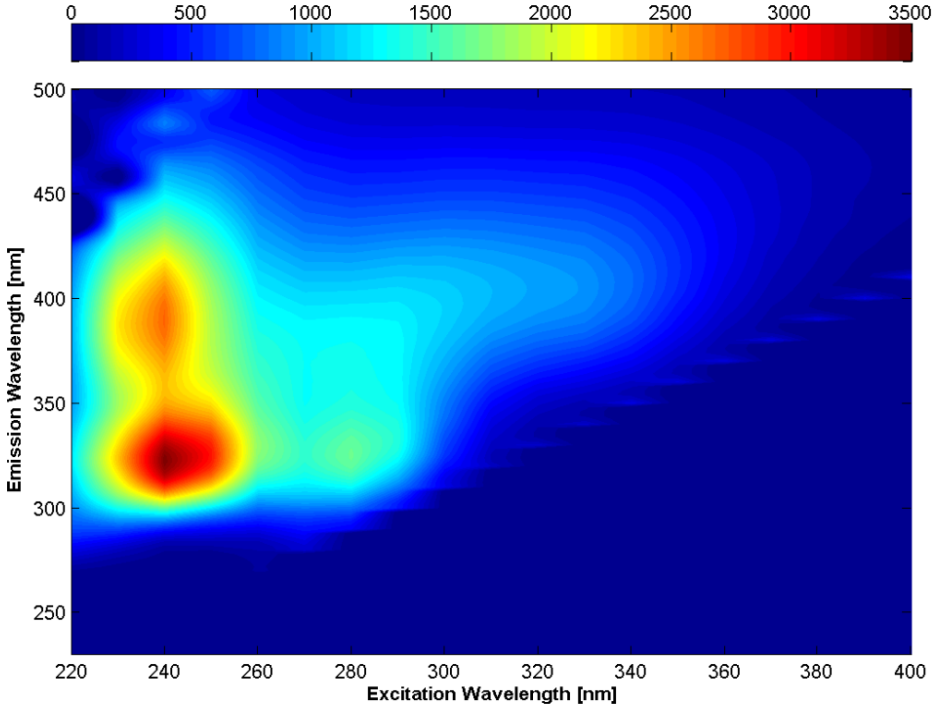


Figure 66 EEM fluorescence spectrum of Sample 1.

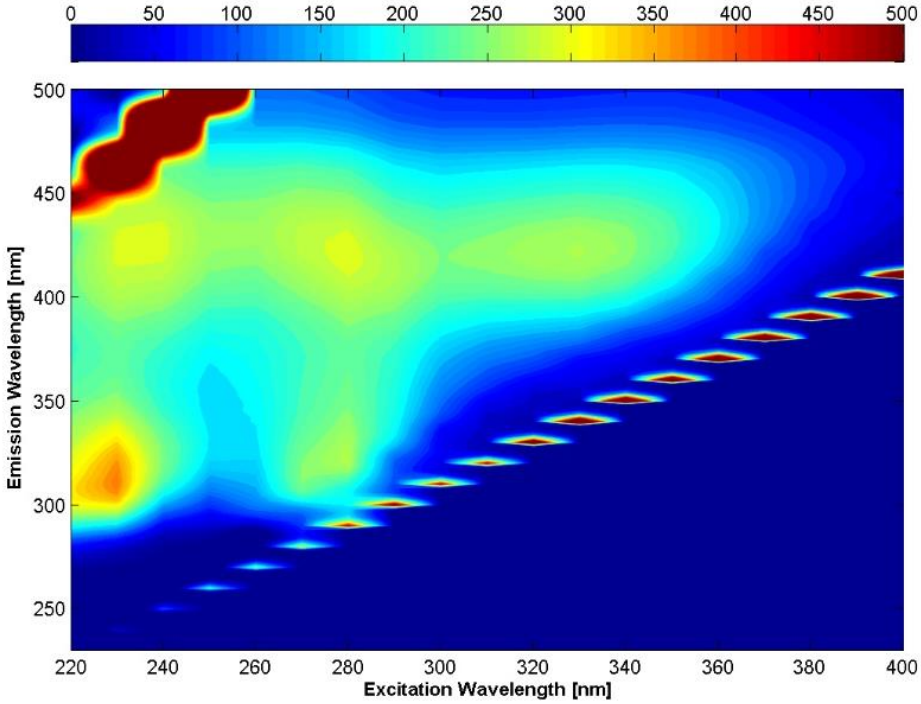


Figure 67 EEM fluorescence spectrum of Sample 2.

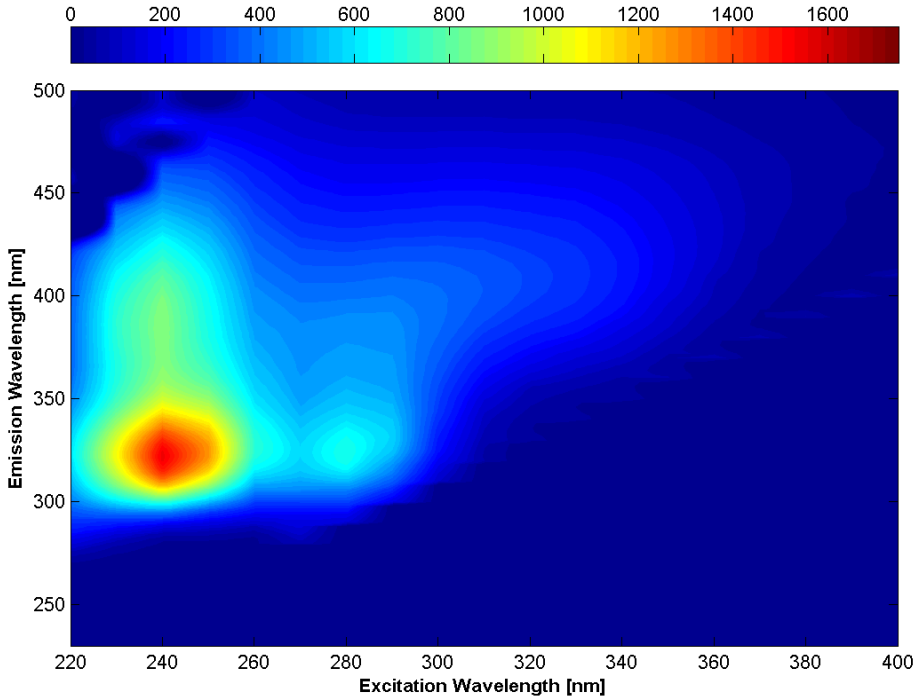


Figure 68 EEM fluorescence spectrum of Sample 3.

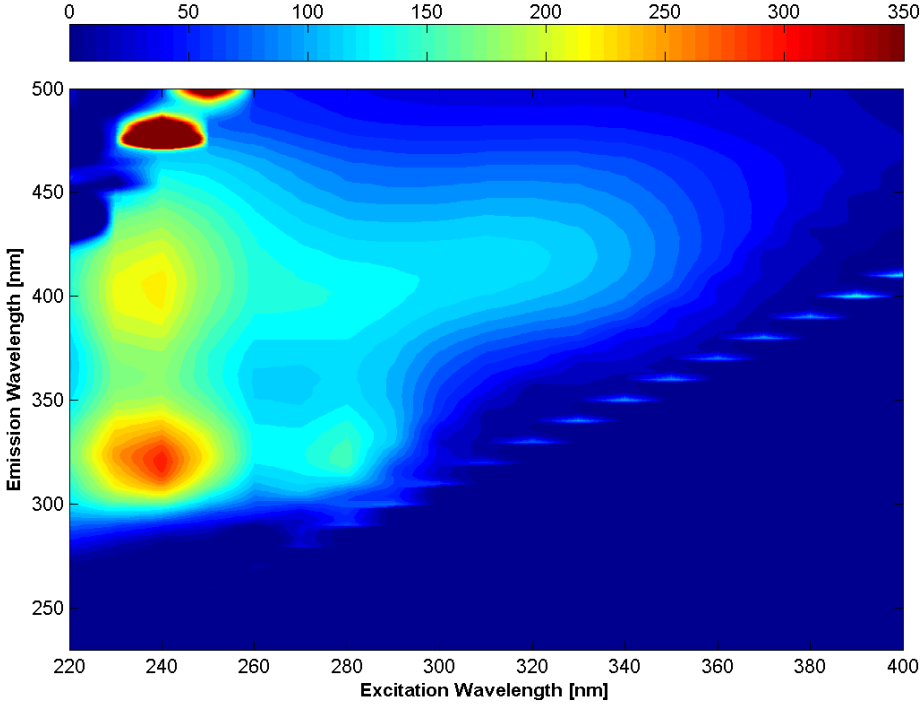


Figure 69 EEM fluorescence spectrum of Sample 4.

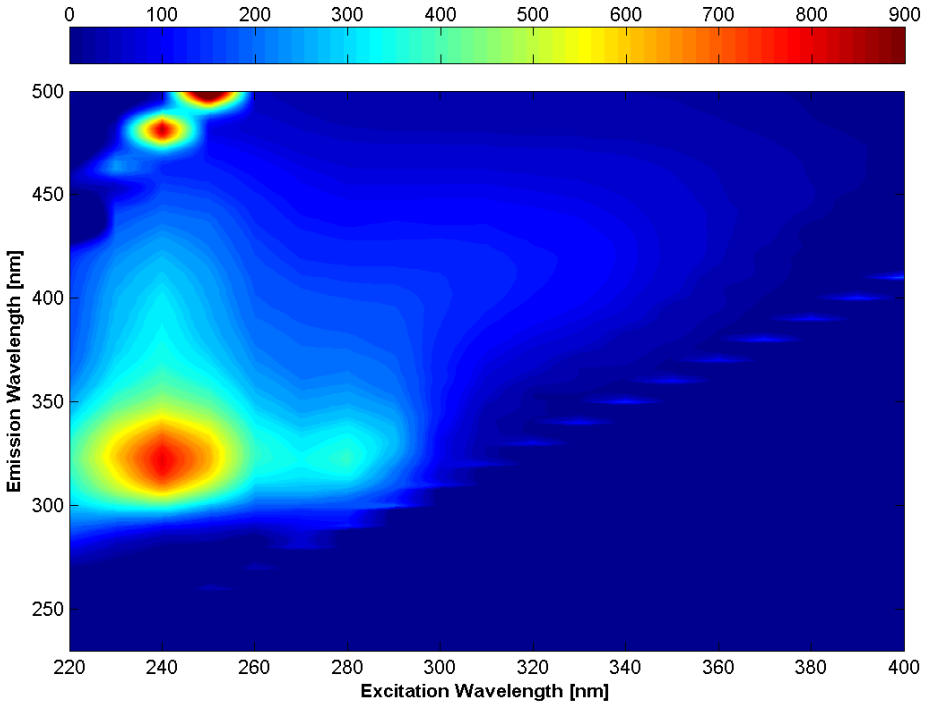


Figure 70 EEM fluorescence spectrum of Sample 5.

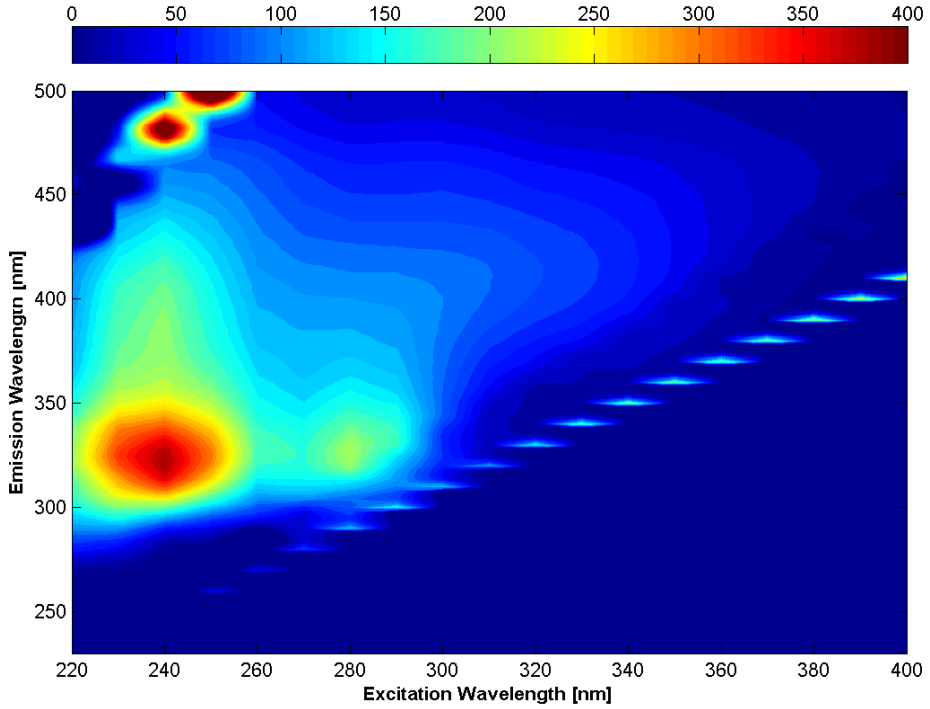


Figure 71 EEM fluorescence spectrum of Sample 6.

Figures 66 to **71** depict the six EEM fluorescence spectra suggesting there is a difference in terms of fluorescence intensity among all the spectra of the bulk aerosol samples. This difference may be due to the different amount of bulk samples collected between the bottom (**Figure 66** and **68**) and up (**Figure 67** and **69**) filters. In spite of the difference in the intensity, the global fluorescence profile is similar among these samples, which suggest that the presence of similar fluorophore structures in all the six cases. As stated before, the significant resemblance between the spectra **Figure 66** and **Figure 68**, **Figure 67** and **Figure 69**, corroborates the idea that the way the filter is placed in the sampler has more influence in the amount and type of collected aerosols than the location of the sampler in the city of Aveiro. Moreover, it is also possible to verify the similarity between the spectra in **Figure 70** and **Figure 71,** which correspond to the aqueous extracts from the filters placed in the sampler in the vertical position in the roof of the STIC Department at the University of Aveiro. The slight different in their intensity may be the result of the wind direction, suggesting that the wind carried out more particles towards filter 6, in **Figure 71**.

V

Conclusions and suggesting to future work

The dispersion of airborne particles containing biological material has a significant effect on the environmental processes and human health, since it contributes to the modification of cloud coverage and can lead to cardiovascular diseases, as well as to allergic and infectious responses in people. Consequently, there is a growing interest in the study of the impact of biological aerosols, although it is still difficult to have an estimate of the total amount of bioaerosols in the air. One method of studying bioaerosols is by detecting the presence of amino acids in atmospheric particles and, therefore, these biomolecules have been considered an important proxy of bioaerosols. One technique that can be used to assist in the study of the presence of amino acids in atmospheric particles is liquid chromatography.

For assessing the separation of amino acids, four different columns were used in an attempt to find the one more suitable for a gradient separation, in which the factor that changes is the organic solvent content of the mobile phase. Although there was a significant difference in the retention time of the individual amino acids in the Mixed--Mode WAX-1 column, that slight separation only occurred for the elution with a buffer concentration of 20mM, a pH = 4.00 and an organic solvent content (MeOH) of 20% (V/V). Furthermore, for the other buffer concentrations (50mM, 100mM and 200mM), it was not possible to ensure the elution of the analytes, since these appeared on the column void volume. Therefore, the Mixed-Mode WAX-1 column was not chosen for performing the liquid chromatography separation on the collected aerosol samples.

Considering the Mixed-Mode HILIC-1 column, the results revealed that the organic solvent concentration had no influence in the elution of the aspartic acid, lysine, glycine and tryptophan, when a 50mM buffer concentration and a pH of 5.45 are applied, either when the samples are dissolved in the same solution that the mobile phase or in a solution not containing any organic solvent. The results also showed that the organic solvent concentration did not have a relevant influence in the elution of the amino acids when the mobile phase had no buffer, the pH was 2.98 and the samples were dissolved only in acidified water: there was no significant difference in the elution of the aspartic acid, lysine glycine and tryptophan, despite of the substantial variation of the organic solvent content in the mobile phase. Thus, the Mixed-Mode HILIC-1 column was not chosen for the liquid chromatographic separation of the collected samples.

Regarding the Luna HILIC column, it was possible to conclude that the preparation procedure of the samples before the chromatographic separation is a factor with crucial influence for the aspartic acid, glycine and lysine amino acids, affecting the

shape of the peaks but not the retention time of the amino acid: if the samples were not taken to the sonication bath, the chromatograms would show more than one peak, revealing a possibility of a lack of solubility of the samples in the mobile phase, which introduced another influence in the analysis – the time gap between the dissolution of the amino acids and the injection moment. For a mobile phase with pH 4.00, 15% (V/V) of 5mM buffer (ammonium acetate and acetic acid) concentration, 76.5% (V/V) of ACN and 8.5% (V/V) of acidified water (pH 3.61, acidified with 0.1% (V/V) formic acid), the retention times of the four amino acids (aspartic acid, glycine, lysine and tryptophan) were very close to each other, which does not guarantee a successful differentiation of the amino acids in a mixture.

With the Luna C18 column, only gradient elution modes were tested for the 20 common amino acids. Bearing in mind this stationary phase is only influenced by the organic solvent content since it only works with one functional mode, RP, the gradient conditions were chosen and applied according to the organic solvent effect. With the results from several gradient elutions, it was possible to achieve a method capable of separating eleven amino acids in a mixture with twenty, suggesting this could be an effective elution for the goal of separating the amino acids in the collected aerosol samples. Therefore, the Luna C18 column was chosen to be used for the chromatographic separation of the collected aerosol samples, applying the following gradient of ACN and acidified water: 0 – 2 min: 5% ACN, 2 – 8 min: 5 – 2 % ACN, 8 – 16 min: 2% ACN, 16 – 20 min: 2 – 20 % ACN, 20 – 35 min: 20 – 35 % ACN.

In order to preserve the representativeness of the natural process of samples deposition, the aerosols samples were collected via passive sampling procedures: three samplers were placed in two different locations in the urban area of Aveiro. The liquid chromatography results revealed similar retention times and broad peaks in all the six collected samples, not allowing an effective and individual identification of the amino acids in the collected samples. Still, considering the retention time of the compounds and the chromatograms obtained in the preliminary tests, there is the chance of having lysine, histidine, arginine, glycine, serine, asparagine, glutamine, alanine, threonine, aspartic acid, glutamic acid, cysteine, proline and valine in the samples. From the chromatograms and the EEM fluorescence spectra, it is possible to observe a similar shape of the peaks and signals, respectively, in the results of all the six samples. However, it is notable the intensity of the chromatographic and fluorescent signal of sample number 2. In fact, filter number 2 had more organic matter since it suffered an intense bird activity. According to

the EEM fluorescent spectra and the chromatograms, there is a pattern regarding the sampling: filters placed in the same spot considering the samplers (up or bottom) have similar shapes and intensities both in the chromatograms and the EEM spectra. Therefore, it is possible to conclude that the filter being placed bottom or up regarding the sampler has more influence than the location of the sampler in Aveiro (near Glicínias Plaza or at the University). Samples collected from up filters had higher intensity values, revealing that up filters collected more matter, both organic and inorganic. Samples 5 and 6 were not placed up or bottom but left or right, since the sampler was placed in the vertical position. Although these two samples showed very similar profiles regarding retention time, peak shape and intensity (both in liquid chromatography and EEM fluorescence spectroscopy), there was a slight difference in the spectra due to the variation of the wind during the sampling month, causing the filter 6 to collect more matter.

In conclusion, although it was possible to assume the presence of some amino acids in the collected aerosol samples at the city of Aveiro, their match to an individual peak was not achieved. Furthermore, the separation of the amino acids was only possible with one out of four tested stationary phases, revealing that the most efficient functionality to separate these biomolecules is the RP mode.

Further studies should be performed in order to improve a gradient elution mode concerning not only the organic solvent content but also the pH of the mobile phase, in order to get an efficient separation in columns with more than one functional mode, since amino acids species are influenced by the pH and the elution is affected by the organic solvent content. This work may turn into a basis for studying amino acids in real environmental samples with simple chromatographic techniques, once the compounds are separated and detected (preferable without derivatization).

VI

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The figures of all the amino acids molecules shown in this Thesis work were designed with the software **ChemBioDraw Ultra 14.0**.

Webgraphy:

Current Weather in Aveiro, Portugal:

http://climetua.fis.ua.pt/legacy/main/current_monitor/cesamet.htm